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Founded 1924

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ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Section A Pathology

CONTENTS

Vol. 82 A. Fasc. 1 1974

PRINTED IN DENMARK VALD PEDERSENS BOGTRYKKERI A S COPENHAGEN

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ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA
Section A Pathology

CONTENTS

Vol 82 A. Fasc. 2 1974

Continued on page 3 of cover

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CONTENTS

Vol. 82 A. Fasc. 2. 19[illegible]

Continued on 3[illegible]

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Continued from page 4 of cover

CONTENTS

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Section A · Pathology

CONTENTS

Vol 82 A Fasc 3 1974

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ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA
Section A: Pathology

CONTENTS

Vol 82 A Fasc. 4. 1974

Continued on page 3 of cover

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CONTENTS

Continued from page 4 of cover

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA
Section A: Pathology

CONTENTS

Vol 82A. Fasc 5 1974

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CONTENTS

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Acta path. microbiol scand Section A 82, 1-6 1974

CHROMOSOME AND NUCLEAR DNA STUDY OF A UTERINE ADENOCARCINOMA AND ITS METASTASES

INGRID GRANBERG, SUBHASH GUPTA, INGEMAR JOELSSON and ERNST SPRINGER

The Department of Pathology of Karolinska Institutet at Huddinge Hospital, the Department of Obstetrics and Gynecology of Karolinska Institutet at Sabbatsberg Hospital Stockholm, Sweden and the Department of Pathology, University of Freiburg, West Germany

A uterine adenocarcinoma was studied by both chromosome and nuclear DNA analysis The primary tumour was characterized by a pseudodiploid stemline, and one of the metastases in the mesenterium revealed a hypotetraploid mode and also a smaller near triploid peak

In a study of carcinoma of the uterine corpus, the findings at hysteroscopy (*Joelsson et al* 1971) concerning the intrauterine extent of the tumour are correlated to its surface and myometrial extension as determined by histopathological techniques In cases with stage I of the disease (FIGO classification, 1971), the frequency of lymph node metastases in the pelvis is evaluated In addition, endometrial cancers are analysed cytogenetically Among the cases hitherto studied one patient was of special interest since a cytogenetic analysis was available from both the primary and the secondary growth

MATERIAL AND METHODS

This patient was a 64 year old previously healthy woman who presented herself with a 6 months history of bleeding and discharge The tissue specimens obtained at fractional curettage from the corpus compartment contained fragments of moderately to low differentiated adenocarcinoma No carcinoma was detected in the portion of the specimen taken from the endocervix Hysteroscopy revealed a large papillomatous tumour which did not extend distal to the internal os The depth of the uterus was 8 cm at bimanual palpation the cervical part of the uterus was normal and the corpus moderately enlarged The para uterine tissue was normal and no masses could be palpated in the true pelvis Pulmonary roentgen examination did not reveal any lung metastases In accordance with FIGO classification the case was consequently classified as the clinical stage I of corpus carcinoma

The patient was referred to surgical treatment and subsequent radiotherapy Total hysterectomy and bilateral salpingo oophorectomy were performed including the extirpation of an enlarged para aortic lymph node and a small mesenteric metastasis which was observed during surgery The uterus was enlarged and rather soft Pelvic lymph adenectomy was not performed because of extensive adhesions

Histological examination of the hysterectomy specimen revealed that the tumour extended ly into the myometrium clumps of

Received 8 vi 73 Accepted 2 vii 73

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TABLE 1 *Chromosome Counts in the Different Specimens*

Specimen	No of counts	No of chromosomes/cell																						
		35	39	40	42	43	44	45	46	50	63	64	78	80	82	83	84	86	88	89	90	92	93	97
I	46	1	1	3	2	8	4	4	18	2									1	1		1		
II	2								2															
III	1								1															
IV	18								2		1	1	1	1	1	2	1	2	1	1	1		1	1

TABLE 2 *Chromosome Characteristics in Relation to the Normal Female Karyotype*

Specimen	No of chromo somes/cell	Chromosome type										No of analy
		A1	A2	A3	B	C	D	E16	E17 18	F	G	
I	39	—1			—1	—2	—1			—1	—1	1
	40					—3	—3					1
	40			+1	—1	—2	—2		—1		—1	1
	42			+1	— 1		—1		—1	—2		1
	43			+1	—1		—1					2
	43			+1		—1	—2		—1			1
	43			+1	—1	—1	—2					1
	44						—2					1
	45			+1		—1	—1					1
	46			+1			—1					5
II	46			+1			—1					2
III	46					+1	—1					1
	46			+1			—1					1
	63	+1	+1	+2	+2	+5	—1	+1	+2	+2	+2	1
	64	+1	+1	+2	+2	+7		+1	+2	+1	+1	1
	82			+1	—1	+2	—7	—1	—1	—1	—2	1
	86			+1		—1	—4			—1	—1	1
	88			+1	—1	—1	—3					1
	89			+2		—2	—3					1
	90			+2		—2	—2					1
	127			+2		—1	—3	+3	+6	+1	+4	1

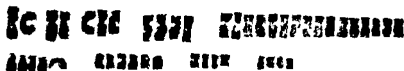
were located in several of the lymphatic vessels near the serosal surface of the uterus The para aortic lymph node and the metastasis from the mesenterium both had tumour growth of the same histology and degree of differentiation as the primary carcinoma

Chromosome analysis was performed on tissue from the large primary tumour (specimen I) a small intramural tumour in the isthmic region separated from the intracavitary tumour (specimen II), the para aortic lymph node (specimen III) and the mesenteric metastasis (specimen IV) The *in vivo* squash method was applied (*Granberg* 1971) In addition part of the material was prepared according to the conventional air dry method and fixed in Carnoy s solution Examination *in the phase contrast microscope* failed however to reveal metaphases suitable for banding analys The Carnoy fixed specimens from the primary tumour and the mesenteric metastasis were used f DNA analysis The smears were subjected Feulgen hydrolysis and dyed by acriflavine for fluorescence cytophotometry (*Bohm & Spre* 1968) The DNA measurements were carried by a Leitz MVP I microscopephotometer comb with *an automatic data processing system (Sp ger & Bohm* 1971)

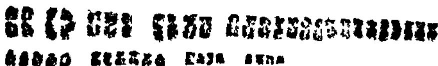
RESULTS

As is obvious from the findings record Table 1, a variable number of metap was found in the different preparatio

2n-46



2n-46



2n-90

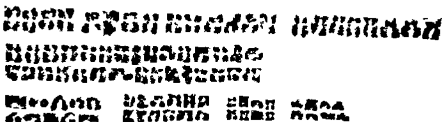


Fig 1 a Specimen I stemline karyotype +1A and —1D b Specimen IV, pseudodiploid karyotype, +1A and —1D c Specimen IV, hypotetraploid modal karyotype, +2A, —2C and —2D (in relation to the normal te raploid chromosome set)

specimen I, in which 46 cells were counted, chromosome number 46 predominated in the diploid modal population, another smaller peak (8 counts) being found at 43, only 3 cells had tetraploid hypotetraploid counts In specimens II and III only single metaphases were found, all containing 46 chromosomes Unlike the mainly diploid-near diploid populations in specimens I III, specimen IV had counts predominantly in the tetraploid region, 2 cells with 46 chromosomes were found, and 4 near triploid cells with counts between 63 and 80

Karyotype analysis was performed in 26 of the 67 metaphases (Table 2 and Fig 1) The stemline was defined at 46 a total of 9 cells in the different specimens having an identical karyotype These cells had gain of one A3 and loss of one D chromosome The variant cells in the diploid region also pre- nted these deviations together with a spread ı and loss in group C and losses in groups 17 18, F, and G

two cells in specimen IV containing 63 and 64 chromosomes, respectively, were karyotyped in relation to the normal diploid karyotype and showed gains and losses similar to those observed in the diploid modal population, namely gain in A3 and loss in D The tetraploid modal population in this specimen, analysed in relation to the normal tetraploid chromosome set, also revealed deviations mainly concentrated to groups A3, C and D

An analysis of the deviations in relation to the mean karyotype in the different chromosome groups (Table 3) shows that all specimens—except the single cell analysed in specimen II—had gain in group A3 and loss in group D In the near-diploid cells, none of the remaining chromosome groups showed deviations exceeding 0 5 (the level of a statistically significant gain or loss of chromo some)

The DNA distribution patterns in specimen I and IV are presented in Figs 2 and 3 The 2c line in each of the histograms was computed from the mean fluorescence values of the leucocytes as an internal reference va-

Fig 2 Feulgen DNA histogram of specimen I n = number of nuclei, FU = fluorescence units (= relative DNA content), 2c, 4c, 8c = DNA content corresponding to a diploid, tetraploid octoploid chromosome set

Fig 3 Feulgen DNA histogram of specimen IV For all other details, see Fig 2

TABLE 3 *Mean Differences between Observed and Expected Values*

Specimen	Mean karyotype	Chromosome type A1	A2	A3	B	C	D	E16	E17 18	F	G	No of karyotypes
I	43,5		+0 1	+0 9	—0 2	—0 1	—1 1	+0 1			+0 1	15
II	46,0			+1 0			—1 0					2
III	46					+1	—1					1
IV	46 0			+1 0			—1 0					2
	63,5	+0 2	+0 2	+1 2	+0 5	—0 1	—2 8	+0 2	+0 5			2
	87,0	+0 2	+0 2	+1 6		+0 9	—3 2		+0 2		—0 2	5
	127	—0 5	—0 5	+1 5	—1 0	—5 2	—4 6	+2 5	+4 5		+3 0	1

lue for each tumour to indicate the DNA content of diploid cell nuclei In this context, therefore, the terms diploid, tetraploid, etc do not refer to the chromosome number directly but to the DNA content to be expected from a normal diploid, tetraploid etc set of chromosomes Consequently, a G phase or mitotic figure of a diploid nucleus will have a tetraploid DNA content (4c) A marked peak in the diploid region and a corresponding small doubling peak in the 4c region is seen in specimen I (Fig 2) In contrast to this, the DNA histogram in specimen IV (Fig 3) exhibits a well defined peak in the 2c region, possibly including a number of normal lymphocytes, and an additional grouping in the 3c area, with doublings in the 6c and 12c regions

DISCUSSION

A majority of the uterine adenocarcinomas exhibit a stemline within the diploid region (*Atkin* 1969 *Baker* 1968 *Wakonig Vaartaja* 1963) In the present case, 94 per cent of the counts from the primary tumour were in the diploid region (Table 4) Obviously the situation is similar in the separate tumour near the isthmic region and in the metastasis of the para aortic lymph node though only a few cells were analysed The 3 near tetraploid counts in the primary tumour possibly represent a polyploidization of the mode, as frequently found in malignant tumours This range of chromosome counts differs from that in the mesenteric metastasis In the latter (specimen IV), the counts were spread within the 2x to 6x regions and, in accordance with the definition, the modal population is tetraploid, with 61 per cent of the counts within this region Also, single cells in the 3x region indicate the apperance of an additional small population, not observed in the primary tumour Similar changes in the karyotypes and mean chromosome deviations make it reasonable to believe that the latter developed by doubling of the mode and later chromosome loss

The chromosome analysis agrees with the DNA measurements DNA histograms of the primary tumour and the metastasis show different patterns, corresponding well to the numerical findings in the two specimens

The karyotype deviations resemble those described in earlier studies (*Baker* 1968), trisomi for a chromosome C or some other group commonly being the only apparent deviation in uterine adenocarcinomas These small chromosome changes are dissimilar to those seen in malignant tumours at other

TABLE 4 *Percentage Distribution of the Counts in the Different Ploidy Regions*

Specimen	Ploidy region 2x	3x	4x	6x
I	94		7	
II	(100)			
III	(100)			
IV	11	22	61	6

sites, for example in carcinoma of the *cervix*, where deviations are usually exhibited by several chromosome groups and marker chromosomes are frequent

The shift to a tetraploid mode in specimen IV is noteworthy, though it seems to be confined to the ploidy level The mean karyotype deviations engage much the same chromosome groups (A3 and D) as in the near-diploid population This indicates that the few triploid cells as well as the tetraploid modal population in specimen IV emerged from the pseudodiploid *stemline in the primary* tumour The result of the chromosome analysis in this tumour does not contradict the opinion that different regions of a tumour and its metastases usually belong to the same clone (*Atkin* 1969) Since only a few reports on the chromosome picture in both primary and secondary tumours are available, the importance of the ploidy shift in this case is still obscure A chromosome analysis of two highly malignant sarcomas at other sites and their metastases did not reveal any change in ploidy (*Granberg & Mark*, manuscript 1973) Measurements on malignant tumours in the lung (*Seidel & Sandritter* 1963), breast, stomach and sigmoid (*Böhm & Sprenger* 1971) reveal a DNA distribution pattern similar to that seen in the metastases It has earlier been suggested that adenocarcinomas of the uterine corpus with a triploid or tetraploid mode exhibit a less favourable prognosis than the carcinoma with a near-diploid mode (*Atkin* 1969) A rough survey of the literature reveals that about 90 per cent of the uterine carcinomas are characterized by a near diploid stemline Clinical experience indicates that corpus carcinomas remain localized for long periods and no correlation has been demonstrated between duration of symptoms and survival rate (*Joelsson et al* 1973) The diploid chromosome and DNA distribution in the primary tumour should favour a good clinical prognosis It is not clear what biological significance should be attached to the ploidy shift in the metastasis and to the appearance of a small triploid population with doubling peaks The patient is well and without any sign of residual or recurrent disease after one year

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This work was supported by the *Swedish Cancer Society*, Project No 126 and 593

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FUNCTIONAL STRUCTURE OF THE RAT LIVER

Influence of Different Types of Preparation

POUL FAARUP, MOGENS JORGENSEN and ANDERS HOLM N ISEN

The University Institute of Pathology
and the Institute of Pathology, Bispebjerg Hospital Copenhagen

The functional structure of the normal rat liver was investigated in rapidly frozen freeze-dried tissue The mean vascular volume of the liver was 27 per cent contrary to conventionally fixed tissue, in which this volume was 7 per cent In the freeze dried tissue the sinusoids were systematic, orientated radially out to the periphery of the lobule The bile capillaries were readily apparent contrasting to those of the delayed frozen or fixed tissue A significant intracellular oedema of he liver cells was found to appear a few minutes after the cutting off of the blood supply

The structure of biological tissue is frequently fundamentally changed shortly after the cutting off of the blood supply to the organ This phenomenon has been found especially pronounced in the kidney (e g Hanssen (1960)) Similar investigations of the liver have not been performed previously Examination of the functional structure and of the rapid *post mortem* changes found in this organ—as revealed in freeze dried sections compared with conventional preparation—has been the task of the pres nt study

MATERIALS A D METHODS

Liver tissue from white ... weighing 150 200 g was used in the pre... ...ly Three groups of rats were investigated

Group I In 4 Nembut... ...etized normal rats a part of the right l... ... measuring 1 2

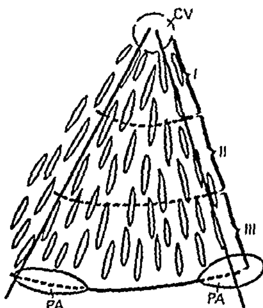


Fig 1 In the scheme a part of the liver lobule has been divided into a central zone (I) close to the central vein (CV), an intermediate zone (II), and an peripheral zone III close to the portal area (PA In this way the vascular and parenchymal volumes of the fractions were studied by planimetry in the rapidly freeze-dried and in the fixed tissue

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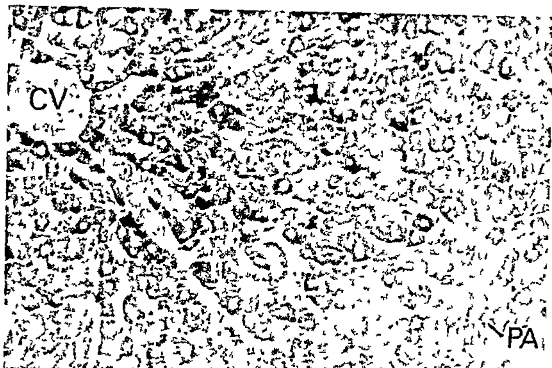


Fig 2 Instantly frozen freeze dried liver tissue in which the s nı so ds are found to be open rad at ng out from the central vein (CV) to the periphery of the l ver lobule Near the central ve n the d ameter of the sinuso ds is larger than the diameter of the ram fications per pherally in the lobı le The vascular volume of the tissue is much b gger than in the delayed frozen or fixed spec mens (*cf* F gs 3 and 4) Due to the stain applied the nuclei of the l ver cells are unsta ned (Freeze dr ed t sue sta ned by osmic acid vapour × 540

cm in diameter was rapidly isolated and frozen in isopentane at about –165° C Care was taken to it traumatic injury to tl e tissue to be investi Sections of 6 8 micron in thickness were cut a cryostat and freeze dried Dur ng the freeze y ng the sections were stained by osmic acid vapour as previously described (*Faarup et al* (1971) The sections were later mounted in paraf fin oil the cover glass being sealed with DPX

Group II In 4 normal rats sim lar l ver t ssue as used in Group I was frozen in isopentane at –165° C 2 minutes after the rats were decapitated The liver tissue was furtl er prepared as described in Group I

Group III In 3 normal rats sim lar liver t ssue was conventionally f xed in neutral formalin and paraffin imbedded The h stological sections were stained by hematoxyl n and eos ne

Methods for Quantitati e Es mat on of the Early post morter (hanges r Liver Tissue

In the rats from grou d p III 4 lobules from each rat liver we concern ng tl e percentage vascular and al volume in the central intermediate and peripheral third of the lobule as seen in Fig 1 This quant f cat on was done by plan metry from draw ngs made by means of d apositives of the lobules

In both the instantly frozen freeze dried tissue and in the fixed t ssue the number of cell nuclei per square mill meter was counted in tl e d fferent zones of the l ver lobule (Fig 8)

Bes des the diameter of the bile cap llaries was measured in photographs as well as by means of an eyepiece micrometer

RESULTS

1 The vascular volume of the liver lobule In the instantly frozen, freeze dried tissue (Fig 2), the vascular volume of the liver lobules were high in contrary to the findings in the freeze dried tissue, in which the freezing of the tissue was delayed for 2 min (Fig 3), as well as in the formalin fixed tissue (Fig 4) The mean vascular volume of the total

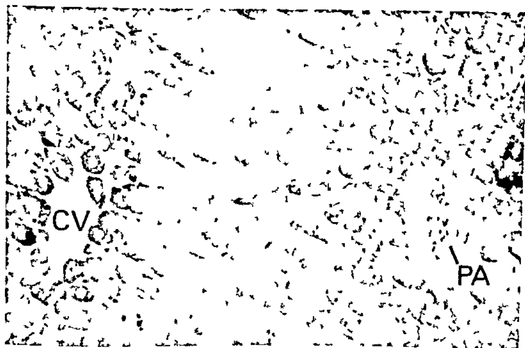


Fig 3 Fr
tion of th
erythrocyt
o ds is obscured Besides the osm ophil a of tl e parenchyma is d minsl ed as compared with instantly frozen t ssue I in ng the s nuso ds some l pophages are seen in wh ch osn opl il c gran iles are present in the cytoplasm (arrows) (Freeze dried t ssue stained by osmic acid vapo ir × 540) Abbreviat ons as in Fig 2

l ver lobule was 27 per cent in the instantly frozen t ssue (Fig 7) In the fixed tissue the mean vascular volume was 7 per cent That the difference in the vascular volumes of the two preparations is mainly due to the early *post mortem* changes is seen from the rather similar appearance of the delayed frozen freeze-dried tissue and the conventionally fixed tissue (Figs 3 and 4)

In the instantly freeze dried tissue the vas cular volume of the central intermediate and peripheral zone of the liver lobule was found to be rather equal (Fig 7) However the sinusoids of the central zone of the liver lo bule close to the central vein was quite con stantly of a greater diameter than that of the ramified sinusoids in the intermediate as well as the peripheral zone of the lobule (F g 2) Equally the vascular volume in fixed tissue was identical in the central the intermediate and the peripheral zone (Fig 7)

2 The parenchymal structure of the liver lobule in freeze dried tissue

A Bile capillaries (canaliculi) The bile cap llaries were well demonstrated in the in stantly frozen parenchyma (Fig 5) The diameter was about 1 3 micron the bile capil laries mostly being more easily identified close to the *portal area* than more centrally in the liver lobule due to the slightly larger diame ter In the delayed frozen freeze dried tissue the bile capillaries were smaller and often difficult to identify as was the c se in the fixed tissue (*cf* Figs 5 and 6)

B Post mortem parenchymal volume changes in the liver In the liver lobules in

Fig 4 Fixed tissue in which the morphology of the liver lobule is quite similar to the delayed frozen tissue in Fig 2 *as regards the collapsed sinusoids and oedema of the liver cells* (*cf* Fig 8) No radiate course of the sinusoids can be *identified* (H and E × 540) Abbreviations as in Fig 2

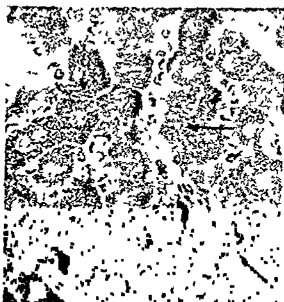


Fig 5 Instantly frozen, freeze dried tissue in which the bile capillaries are found to be open and well identified in this preparation (arrows) (Preparation as in Fig 2 × 825)

which the vascular volume was quantitatively determined the number of liver cell nuclei from identical areas were counted As the number of nuclei were constantly bigger in the instantly frozen, freeze dried tissue, and as the vascular volume was constantly smaller in the fixed specimens, it can be concluded, that *a significant intracellular oedema was present as a post mortem change in the tissue (Fig 8)* Equally, in the freeze dried tissue in which the freezing was delayed for 2 minutes, intracellular oedema was present in the liver cells (Figs 3 and 6) In the instantly frozen, freeze dried tissue the cell counts were found to be identical in the central, the intermediate and the peripheral zone of the liver lobule Thus the size of the liver cells in the three fractions were found to be identical (Fig 8) This was equally found to be the case concerning the fixed tissue

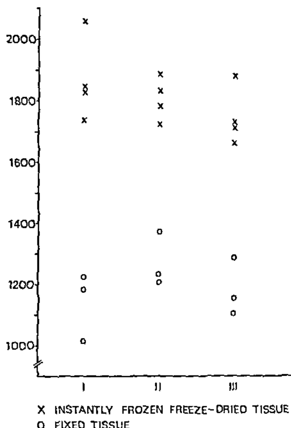


Fig 8 Number of liver cell nuclei counted per square millimeter in instantly frozen freeze dried and in fixed tissue Each value comprises 4 liver lobules from one rat The number of liver cells per square millimeter is much bigger in the freeze dried tissue than in the fixed tissue in spite of the vascular volume being biggest in the freeze dried tissue (*cf* Fig *7*) This indicates that a significant intracellular oedema of the liver cells is present in the [illegible] specimens Ordinate Number of liver cells *per square millimeter* (*cf Fig 1*)

fixed tissue indicate, that occlusion of the bile capillaries does occur to some degree briefly after the cutting off the blood supply of the liver

In conclusion, this study has demonstrated that in the normal rat liver pronounced changes in the parenchymal and in the vascular volume take place from the functioning to the fixed state, comprizing a significant decrease in sinusoid and bile capillary diameter and the appearance of a great intracellular parenchymal oedema originating in the early *post mortem* period

This work was supported by '*Statens almindelige Videnskabsfond*', '*Foreningen til Hjertesygdommenes Bekæmpelse*', *P A Brandts Fond* and "*Kong Christian Den Tiendes Fond*

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Acta path microbiol scand Section A 82, 13–20, 1974

FUNCTIONAL STRUCTURE OF THE CIRRHOTIC RAT LIVER

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In rats with carbon tetrachloride induced cirrhosis the liver was investigated in rapidly frozen freeze dried specimens In the rats to which the administration of carbon tetrachloride was stopped 10 days prior to sacrifice the relative sinusoidal volume was found to be higher in the cryo preparations (mean 24 per cent) compared to formalin fixed tissue (mean 10 per cent) due to vascular collapse and a rapid development of intracellular oedema in the liver cells as a *post mortem* alteration in the formalin fixed tissue This is parallel to previous findings in normal rat livers In the group of cirrhotic rats treated with carbon tetrachloride until 24 hours before sacrifice necrosis of the liver cells was frequently found In this group the relative vascular volume was much lower (mean 14 per cent) than in the rats without necrosis (mean 24 per cent) in the cryo preparations These changes in sinusoidal volume, not visualized in conventional histological preparations are explained by the presence of intracellular oedema of the liver cells caused by the carbon tetrachloride intoxication In this group of rats the bile canaliculi were poorly visualized compared to the rats without recent intoxication It is concluded that the use of an appropriate cryo technique is essential for the evaluation of the vital alterations in the cirrhotic rat liver which is only poorly depicted by conventional histological technique

In a previous study it was demonstrated, that significant morphological changes take place in the early *post mortem* period in the normal rat liver *(Faarup et al* 1973) By the use of an appropriate cryo technique it was shown, that the vascular volume of the liver was much diminished shortly after the cutting off of the blood supply This paralleled the rapid appearance of a significant intracellular oedema of the liver cells In the present study cirrhotic rat livers with and without recent necrosis were investigated in a similar way for changes in vascular and parenchymal structure by the same cryo technique, which depicts the vital conditions more clearly than does the more conventional histological technique

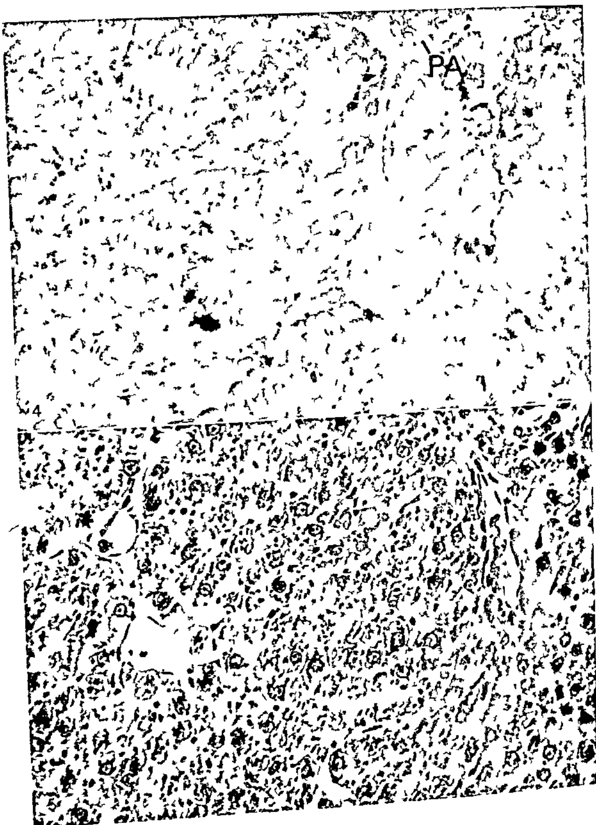
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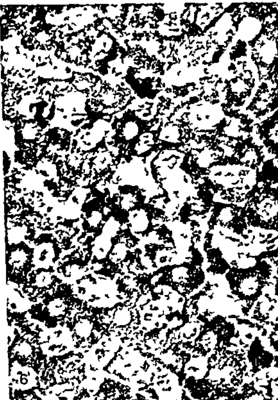
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MATERIAL AND METHODS

White male rats of an initial weight of 150–200 g were used Cirrhosis of the liver was produced according to the method described by *McLean et al* 1969 This method implies simultaneous administration of phenobarbitone and carbon tetrachloride The phenobarbitone seems to act as an inducer of the microsomal enzyme system which metabolizes the carbon tetrachloride and thereby enhances its toxicity (*Garner & McLean* 1969) The drinking water was added 0 05 per cent sodium barbitone Carbon tetrachloride was administered by inhalation twice a week The dosage followed the schedule advocated by *McLean et al* 1969 and a total of 10 inhalations was given

The rats were divided in two groups A and B


PA
4
5



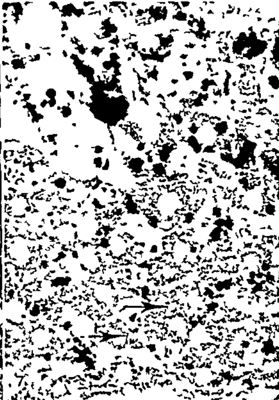


Fig 6 Cirrhotic rat liver without liver cell necrosis (group B) Many bile capillaries are identified in the parenchyma (Arrows) (Freeze dried tissue × 1120)

Fig 7 Cirrhotic rat liver with recent liver cell necrosis (group A) In the necrotic tissue no bile capillaries are seen In the non necrotic tissue only few bile capillaries are identifiable (Arrows) (Freeze dried tissue × 1120)

was found between the livers from group A and B Macroscopically no hemorrhage or necrosis were observed, and no ascites or jaundice were present

Microscopically typical cirrhotic changes were present in all the livers (Fig 1) In addition cytoplasmatic degeneration of the liver cells in the form of vacuolization was frequently recorded most pronounced in the livers from group A (Fig 2 + 3) In the freeze dried sections no fat was present in these vacuoles as evidenced by the osmic acid stain But osmic acid positive material was present in Kupffer cells of both groups of rats (Fig 2 + 4) In group B no necrosis of the liver cells was encountered (Fig 4 + 5), in group A necrosis was a constant finding in most of the lobules or nodules (Fig 2 + 3) As in the normal rat liver the bile capillaries were easily identified in the freeze dried sections from the livers of group B (Fig 6), but quite seldom in the livers from group A in which many recent necroses were found (Fig 7) The relative sinusoidal and parenchymal volumes were determined, and the results appear from Fig 8 It is seen that no

Fig 4 Cirrhotic rat liver without liver cell necrosis (group B) The sinusoids are easily identified all over in the parenchyma Kupffer cells containing osmic acid positive material are scattered throughout the tissue PA portal area (Freeze dried tissue × 540)

Fig 5 Formalin fixed tissue from the same rat liver as in Fig 4 As in Fig 3 the sinusoids are highly collapsed (HE × 790)

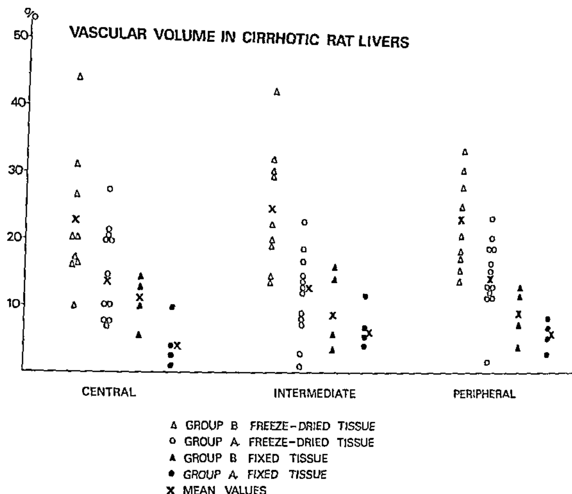


Fig 8 The vascular volume of the cirrhotic rat livers with recent liver cell necrosis (group A) and without liver cell necrosis (group B) in freeze dried as well as in formalin fixed tissue In freeze-dried tissue from the rats of group B the vascular volume was high and rather equal in different zones of the parenchyma In the livers with recent necrosis (group A) the vascular volume was clearly diminished In the formalin fixed tissue the vascular volume was systematically lower than in the freeze dried tissue in both group A and B rats

systematical difference was found in the relative sinusoidal volumes from different localizations (viz central, intermediate and peripheral)

In group A, however, containing recent liver cell necrosis the vascular volume was systematically lower than in group B Besides, it appears from Fig 8, that the relative sinusoidal volume was systematically lower in the formalin fixed tissue compared to the freeze-dried tissue in the livers from both group A and B rats The percentage of parenchymal necrosis in group A ranged from zero to 97 per cent in the different areas The localization was predominantly central or intermediate (Fig 9) No correlation was found between the degree of necrosis and the relative sinusoidal volume in the respective areas quantitatively investigated (Fig 10)

DISCUSSION

The high sinusoidal volume found in the freeze dried sections from the cirrhotic livers of group B without recent necrosis (mean 24 per cent) is very different from the small sinusoidal volume encountered in the formalin fixed tissue (mean 6 per cent) (Fig 8) These values are very close to the figures found in normal rat liver and illustrate the

LIVER CELL NECROSIS

Fig 9 The percentage degree of liver cell necrosis in rats receiving carbon tetrachloride unt l sacr f ce (group A) The necros s was less frequent near the portal areas than in central and intermediate zones of the parenchyma This was the case in both freeze dried and formal n fixed tissue

early *post mortem* changes in the tissue in case of slow fixation as previously discussed (*Farup et al* 1973) Liver cell necrosis was a constant finding in the rats from group A in which carbon tetrachloride was administered until sacrifice whereas no necrosis was encountered in group B in which the carbon tetrachloride treatment was stopped 10 days before sacrifice The percentage of the parenchyma found to be necrotic was almost identical in the cryo preparations and in the formalin fixed material (Fig 9) The relative sinusoidal volume was systematically lower in group A than in group B in the instantly frozen preparations (mean 14 per cent versus 24 per cent) As the only difference between group A and B is the recent administration of carbon tetrachloride to the rats of group A this recent intoxication must be responsible for the difference As no correlation exists between the degree of parenchymal necrosis and the relative sinusoidal volume in the different parenchymal areas in group A ra s (Fig 10), this reduction in vascular volume can not selectively be caused by the necros s Probably it is mainly due to oedema of the liver cells This also explains why the identification of the bile capillaries was very difficult in the livers of group A whereas they were very easily seen in the livers of group B lacking recent parenchymal damage

Degeneration of liver cells including oedema and necrosis is a well known effect of carbon tetrachloride intoxication (*Sherlock* 1968) A quantitative evaluation of the concomitant reduction in relative sinusoidal volume has not previously been made The use of an appropriate cryo technique is essential in this respect as conventional histolog cal technique on account of the early *post mortem* changes secondary to slow fixation blurs this phenomenon

Th s work was supported by grants from Statens alm ndel ge V denskabsfond Foren ngen til Hjertsygdommenes Bekæmpelse , P A Brandts Fond and Kong Christ an den Tiendes Fond

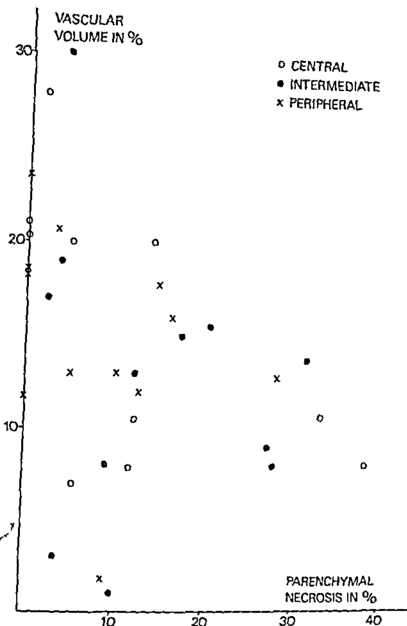


Fig 10 The percentage degree of *necrosis* and the relative vascular volume in identical areas of livers from group A rats in which liver cell necrosis was a constant finding No correlation is found between the two parame ers

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Acta path microbiol scand Section A 82, 21-29, 1974

A STEREOLOGICAL STUDY OF INTRAHEPATIC BILE DUCTS

4 Congenital Hepatic Fibrosis

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The intrahepatic biliary system in congenital hepatic fibrosis has been evaluated by means of stereology and reconstructions in a material from 8 patients The ductal system was found to consist of a characteristic mixture of different ductal shapes including very flattened plate like ducts The arrangement of these ducts is in irregular networks usually organized in separate planes and often completely or incompletely surrounding vessels No change in the composition of the ductal mixture with age was found The biliary system is fundamentally identical in congenital hepatic fibrosis and in infantile polycystic disease It is not possible to determine from the present study whether or not there may be a minor systematic difference between the ductal lesion of the two diseases The pathogenesis is discussed on the basis of the anatomical findings and the flattened ductal shape is considered to be due to a failure in the embryological development of the ducts

Among the causes of portal hypertension in children and young adults, *Kerr et al* 1961 separated a clinico pathological entity which they called *congenital hepatic fibrosis* (CHF) The disease typically presents with hepatosplenomegaly or with bleeding from oesophageal varices in a child with normal liver function Liver biopsy is essential in order to establish the diagnosis and the liver histology is characteristic Septa of mature collagen originate from the portal tracts without interfering with the normal lobular architecture of the parenchyma and the liver cells look normal There is an increased number of longitudinally sectioned bile ducts in the connective tissue (Fig 1)

Received 25 vii 73 Accepted 7 ix 73

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This liver histology was originally described by *MacMahon* 1929 in a paper on congenital anomalies of the liver The patients showing this hepatic histology have later been grouped under the diagnoses congenital hepatic fibrosis and infantile polycystic disease according to clinical picture, renal involvment etc

Considering the importance of liver anatomy—diagnostically and for our understanding of pathogenesis and pathophysiology—it is very surprising that, although a considerable number of papers has been published on these diseases, little has been added to the original anatomical description Neither the fibrous septa nor the vascular and biliary structures have been the subject of intensive studies The bile ducts are very conspicuous in the histological picture and diagnostically probably the most important element The purpose of the present investigation has been to make an anatomical study of these structures

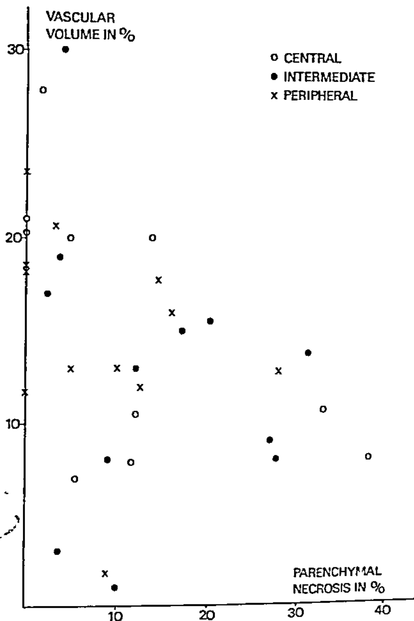


Fig 10 The percentage degree of necrosis and the relative vascular volume in identical areas of livers from group A rats in which liver cell necrosis was a constant finding No correlation is found between the two parameters.

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Acta path microbiol scand Section A 82, 21 29, 1974

A STEREOLOGICAL STUDY OF INTRAHEPATIC BILE DUCTS

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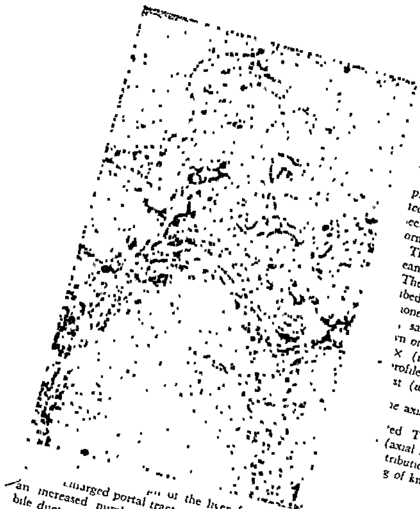
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...enlarged portal tract and fibrous septa with an increased number of longitudinally sectioned bile ducts are seen The architecture of the parenchyma is normal and no inflammatory infiltrates are present (HE × 40)

MATERIAL

...e material consists of 10 liver specimens from 8 ...ients Seven of the specimens are surgical and ...re from autopsies A detailed report of the ...cal, radiological and genetic information about ...atients is beyond the scope of this paper, but ...formation is summarized in Table 1

The statement about the kidneys is based on the result of intravenous pyelography or on autopsy findings In case 4 radiological examination of the kidneys showed both to be enlarged but without signs of polycystic disease

Cases 4 & 7 have previously been published (*Sommerschild et al* 1973)

METHODS

Two hundred to five hundred 5 μm thick serial sections have been made from the paraffin embedded liver specimens The number of sections has been limited by the amount of tissue available Staining was with van Gieson Hansen stain.

The ductal structures have been followed and studied through these series

Three-dimensional reconstructions have been made from selected cases

The method of reconstruction has been described previously (*Jørgensen* 1971 a) In brief, the technique implies transfer of drawings of the serial ...ections to acryl plates which, when glued together, ...orm a transparent block

The biliary system has also been evaluated by ...eans of stereology

The method applied has previously been de...ibed in detail (*Jørgensen* 1973 a) In brief, ...ioned ductal structures (profiles) from ea... ..., sampled at random from one slide, wer... ...n on tracing paper at a total magnification o... × (microscope + drawing device) Each of ...rofiles was numbered and its longest (l) and ...st (w) diameter measured. From these fig... ...e axial ratio ($q = \frac{w}{l}$) of the profile was ...ed The frequency of the different profile ... (axial ratios) is compared with the theoret... ...tributions of axial ratios from random ...g of known geometrical shapes.

RESULTS

The distribution of axial ratios from the 200 randomly selected profiles is given in Table 2...

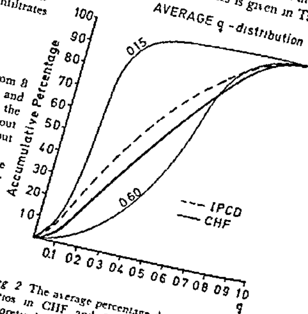


Fig 2 The average percentage distribution of axial ratios in CHF and IPCD For comparison the theoretical distribution for random sections of straight cylinders with q of directrix 0.15 and 0.60 are indicated

TABLE 1 *Case Summaries*

Case no	Sex Female = F Male = M	Hepato splenomagaly	Bleeding from oesophageal varices	Shunt operation	Polycystic kidneys	Siblings affected	Dead	Specimen no	Age at biopsy or autopsy (years)
1	M	+	+	+	+	0	+	1	20
2	M	+	+	+	+	0	+	2	20
3	F	+	+	–	–	0	–	3	8
4	M	+	–	–	– (enlarged)	–	–	4	9
5	M	+	–	–	+	+	–	5A	3
								5B	10
6	M	+	+	+	–	–	–	6	5
7	F	+	+	+	+	–	–	7A	10
								7B	14
8	M	+	+ (small)	–	–	+	+	8	5

\+ = yes
– = no
0 = no information available

and the average distribution is illustrated diagrammatically in Fig 2 For comparison the theoretical distributions of axial ratios from random sections of straight cylinders with q of directrix 0 15 and 0 60 (directrix = the profile from an exact transverse section of a cylinder) are given, as is the distribution found in a material of infantile polycystic disease (IPCD) (*Jorgensen* 1973 b)

In Fig 3 the spread in distribution of q among the specimens is illustrated together with the corresponding spread found in IPCD *

The distribution of w in microns is given in Fig 4 In addition to the average distribution for the total material the distributions of the extremes are shown The distributions in case 1 and case 4 were practically identical

* Case 1 has been omitted from Fig 3 The histological slides showed a very large number of rounded profiles filled with polymorphonuclear leucocytes The acute cholangitis was secondary to a porto-caval shunt operation and was fatal The influence of this cholangitis on ductal morphology makes this case unsuitable for the demarcation of the spread in q distribution found among uncomplicated cases The influence on the average distributions of q and w is only minor and case 1 has not been omitted from these graphs

As may be seen from Fig 5, the average distributions of w in CHF and IPCD are very similar The same applies to the distributions of the extremes in both materials (not shown in the illustrations)

No correlation is found between the distribution of the stereological parameters (q and w) and the existence of portal hypertension as evidenced by bleeding oesophageal varices

The result of the 4 reconstructions is illustrated in Fig 6–13 To summarize the findings, the ductal structures are arranged in planes which are often bent so as to encircle vessels partially or totally The ducts form an irregular network in these planes and their geometrical shape shows a variable degree of flatness The most flattened ducts present as plates called "*ductal plates*"

DISCUSSION

A The Reconstructions

Very few reconstructions of the biliary structures in CHF have been made hitherto *Landing et al* 1973 made reconstructions from 2 cases of CHF They found dilated ducts arranged in a network at the periphery of the portal areas The dimensions of the cystic dilated ducts and the "holes" in the



Fig 1 Microphotograph of the liver from biopsy 7A An enlarged portal tract and fibrous septa with an increased number of longitudinally sectioned bile ducts are seen The architecture of the parenchyma is normal and no inflammatory infiltrates are present (HE × 40)

MATERIAL

The material consists of 10 liver specimens from 8 patients Seven of the specimens are surgical and 3 are from autopsies A detailed report of the clinical, radiological and genetic information about the patients is beyond the scope of this paper, but this information is summarized in Table 1

The statement about the kidneys is based on the result of intravenous pyelography or on autopsy findings In case 4 radiological examination of the kidneys showed both to be enlarged but without signs of polycystic disease

Cases 4 & 7 have previously been published (*Sommerschild et al* 1973)

METHODS

Two hundred to five hundred 5 μm thick serial sections have been made from the paraffin embedded liver specimens The number of sections has been limited by the amount of tissue available Staining was with van Gieson-Hansen stain

The ductal structures have been followed and studied through these series

Three-dimensional reconstructions have been made from selected cases.

The method of reconstruction has been described previously (*Jorgensen* 1971 a) In brief, the technique implies transfer of drawings of the serial sections to acryl plates which, when glued together, form a transparent block

The biliary system has also been evaluated by means of stereology

The method applied has previously been described in detail (*Jorgensen* 1973 a). In brief, 200 sectioned ductal structures (profiles) from each case, sampled at random from one slide, were drawn on tracing paper at a total magnification of 200 × (microscope + drawing device) Each of the profiles was numbered and its longest (l) and shortest (w) diameter measured From these figures the axial ratio ($q = \frac{w}{l}$) of the profile was calculated The frequency of the different profile shapes (axial ratios) is compared with the theoretical distributions of axial ratios from random sectioning of known geometrical shapes

RESULTS

The distribution of axial ratios from the 2000 randomly selected profiles is given in Table 2

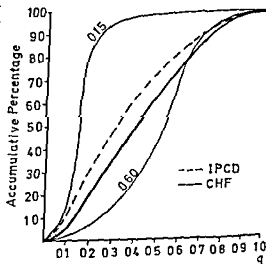


Fig 2 The average percentage distribution of axial ratios in CHF and IPCD For comparison the theoretical *distribution for random sections of* straight cylinders with q of directrix 0 15 and 0 60 are indicated

TABLE 1 *Case Summaries*

Case no	Sex Female = F Male = M	Hepato-splenomegaly	Bleeding from oesophageal varices	Shunt operation	Polycystic kidneys	Siblings affected	Dead	Specimen no	Age at biopsy or autopsy (years)
1	M	+	+	+	+	0	+	1	20
2	M	+	+	+	+	0	+	2	20
3	F	+	+	−	−	0	−	3	8
4	M	+	−	−	− (enlarged)	−	−	4	9
5	M	+	−	−	+	+	−	5A	3
								5B	10
6	M	+	+	+	−	−	−	6	5
7	F	+	+	+	+	−	−	7A	10
								7B	14
8	M	+	+ (small)	−	−	+	+	8	5

+ = yes
− = no
0 = no information available

and the average distribution is illustrated diagrammatically in Fig. 2. For comparison the theoretical distributions of axial ratios from random sections of straight cylinders with q of directrix 0.15 and 0.60 (directrix = the profile from an exact transverse section of a cylinder) are given, as is the distribution found in a material of infantile polycystic disease (IPCD) (*Jorgensen* 1973 b).

In Fig. 3 the spread in distribution of q among the specimens is illustrated together with the corresponding spread found in IPCD.*

The distribution of w in microns is given in Fig. 4. In addition to the average distribution for the total material the distributions of the extremes are shown. The distributions in case 1 and case 4 were practically identical.

* Case 1 has been omitted from Fig. 3. The histological slides showed a very large number of rounded profiles filled with polymorphonuclear leucocytes. The acute cholangitis was secondary to a porto-caval shunt operation and was fatal. The influence of this cholangitis on ductal morphology makes this case unsuitable for the demarcation of the spread in q distribution found among uncomplicated cases. The influence on the average distributions of q and w is only minor and case 1 has not been omitted from these graphs.

As may be seen from Fig. 5, the average distributions of w in CHF and IPCD are very similar. The same applies to the distributions of the extremes in both materials (not shown in the illustrations).

No correlation is found between the distribution of the stereological parameters (q and w) and the existence of portal hypertension as evidenced by bleeding oesophageal varices.

The result of the 4 reconstructions is illustrated in Fig. 6–13. To summarize the findings, the ductal structures are arranged in planes which are often bent so as to encircle vessels partially or totally. The ducts form an irregular network in these planes and their geometrical shape shows a variable degree of flatness. The most flattened ducts present as plates called "*ductal plates*".

DISCUSSION

A. The Reconstructions

Very few reconstructions of the biliary structures in CHF have been made hitherto. *Landing et al.* 1973 made reconstructions from 2 cases of CHF. They found dilated ducts arranged in a network at the periphery of the portal areas. The dimensions of the cystic dilated ducts and the 'holes' in the

TABLE 2 *Percentage Distribution of Axial Ratios (q)*

Specimen no	q intervals									
	0 0 10	0 10 0 20	0 20 0 30	0 30 0 40	0 40 0 50	0 50 0 60	0 60 0 70	0 70 0 80	0 80 0 90	0 90 1 00
1	1 5	6 5	14 0	11 5	16 5	14 5	16 0	11 5	5 0	3 0
2	1 5	21 5	19 0	17 0	14 5	7 5	7 5	6 5	4 5	1 0
3	3 0	15 0	10 0	13 0	14 5	9 5	11 0	15 5	5 0	3 5
4	1 5	13 5	17 5	19 5	11 0	9 0	14 0	8 5	4 5	1 0
5A	7 5	24 0	17 5	10 0	15 0	5 5	10 0	4 5	3 0	3 0
5B	2 5	12 5	11 5	15 0	15 0	14 0	10 5	12 5	3 5	3 0
6	2 5	14 0	19 0	13 5	19 0	11 0	8 5	8 0	3 0	1 5
7A	4 5	16 5	16 5	13 5	13 5	11 0	12 5	6 0	6 0	0
7B	4 0	22 0	18 0	12 5	13 5	10 0	13 0	4 5	1 5	1 0
8	6 5	16 5	11 0	13 0	20 0	12 5	9 0	7 0	4 0	0 5
Average	3 5	16 2	15 4	13 9	15 3	10 5	11 1	8 4	4 0	1 7

network gave the appearance of a "perforated sleeve or cylinder" around the portal spaces The network arrangement of the ducts found in the present investigation is thus in accordance with the result of other studies None of the previously published papers on CHF have made comments on the geometrical shape of the ducts The results of the 4 reconstructions made in the present study, as stated above, is in accordance with the result of the stereological investigation summarized in the following

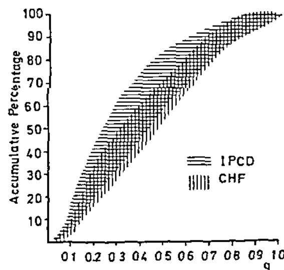


Fig 3 The spread in distribution of axial ratios among the cases of CHF and IPCD Case no 1 has been excluded (see text)

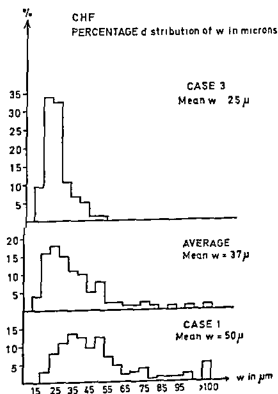


Fig 4 The percentage distribution of *w* in microns. In addition to the average distribution for the total material the distributions of *the extremes* are shown

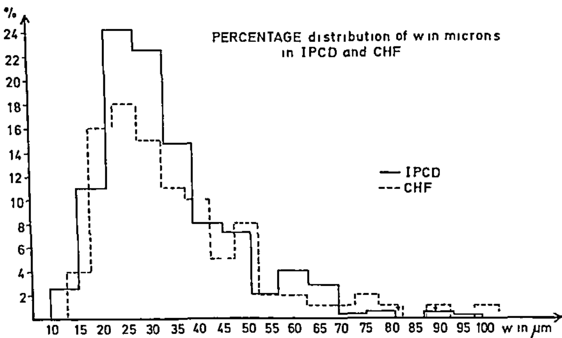


Fig 5 The average distributions of *w* in microns in CHF and IPCD

B The Stereological Investigation

With respect to interpretation in terms of ductal shape of the *q* distribution found (Fig 2), it must parallel that of IPCD (*Jorgensen* 1973 b) In brief, the ductal population must be a mixture of elements approximating to straight cylinders and those of very different shape A substantial part of the cylinders must be somewhat flattened elliptical cylinders with a q of directrix less than 0 10–0 15 Another and significant part of the ducts must approximate to the shape of circular cylinders The different ductal shapes are without preferential width The distribution of *w* (Fig 4) proves quantitatively that the ducts are dilated compared with normal ducts (*Jorgensen* 1973 a) These results are in accordance with the reconstructions

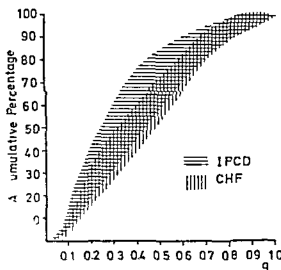
C The Hepatic Lesion in CHF and IPCD

It is generally stated that the hepatic lesions in CHF and in IPCD are indistinguishable as judged from histological slides, and probably identical (e g *Lieberman et al* 1971 and *Blyth* & *Ockenden* 1971) This impression has not been substantiated by means of quantification With respect to the biliary structures, the great similarities between reconstructions from cases of CHF and ICPD support the concept of fundamentally identical lesions *Landing et al* 1973 found changes in 3 cases of IPCD and 2 cases of CHF similar to those found previously in one case of IPCD (*Landing* & *Wells* 1970) The concept of ductal architecture obtained from these reconstructions was confirmed by 3 microdissections from cases of IPCD *Norris* & *Tyson* *1947 investigated 2 cases of IPCD* The reconstruction from the first case showed platelike, dilated ducts encompassing arteries The reconstruction from the second case showed a 3 dimensional network of irregular, tubular ducts with pronounced segmentation The relation between the vessels and the biliary structures was emphasized Apart from 'irregularity and distorsion" no comment is made about ductal shape With respect to the second case it is impossible to judge ductal shape from the graphic reconstruction The

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Specimen no	q intervals									
	0 0 10	0 10 0 20	0 20 0 30	0 30 0 40	0 40 0 50	0 50 0 60	0 60-0 70	0 70 0 80	0 80 0 90	0 90 1 00
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2	1 5	21 5	19 0	17 0	14 5	7 5	7 5	6 5	4 5	1 0
3	3 0	15 0	10 0	13 0	14 5	9 5	11 0	15 5	5 0	3 5
4	1 5	13 5	17 5	19 5	11 0	9 0	14 0	8 5	4 5	1 0
5A	7 5	24 0	17 5	10 0	15 0	5 5	10 0	4 5	3 0	3 0
5B	2 5	12 5	11 5	15 0	15 0	14 0	10 5	12 5	3 5	3 0
6	2 5	14 0	19 0	13 5	19 0	11 0	8 5	8 0	3 0	1 5
7A	4 5	16 5	16 5	13 5	13 5	11 0	12 5	6 0	6 0	0
7B	4 0	22 0	18 0	12 5	13 5	10 0	13 0	4 5	1 5	1 0
8	6 5	16 5	11 0	13 0	20 0	12 5	9 0	7 0	4 0	0 5
Average	3 5	16 2	15 4	13 9	15 3	10 5	11 1	8 4	4 0	1 7

network gave the appearance of a "perforated sleeve or cylinder" around the portal spaces The network arrangement of the ducts found in the present investigation is thus in accordance with the result of other studies None of the previously published papers on CHF have made comments on the geometrical shape of the ducts The results of the 4 reconstructions made in the present study, as stated above, is in accordance with the result of the stereological investigation summarized in the following



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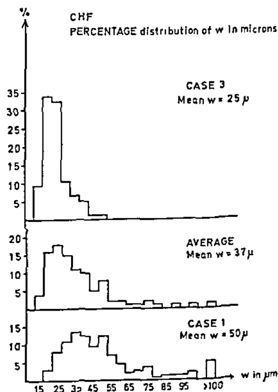


Fig 4 The percentage distribution of *w* in microns. In addition to the average distribution for the total material the distributions of the extremes are shown



12



13

Fig 12 + 13 Reconstruction from specimen no 2 A network of flattened b le ducts is seen Magnificat on × 200 Original block size *7 × 9 × 10 5 cm*

reconstructions made by *the present author* from cases of IPCD (*Jorgensen* 1971 b and 1972) and from the cases of CHF *presented* here show great similarities and no fundamental differences They have features in common with both *the reconstructions* of *Norris & Tyson* and those *of Landing and co workers*

As previously discussed (*Jorgensen* 1973 a) the distribution of the stereological parameters *q* and *w* represents a general characterisation of ductal populations and is thereby applicable for the distinction between different ductal populations With respect to the distribution of *w*, *no significant difference* was found between IPCD and CHF (Fig 5)

Fig 6 Reconstruction from biopsy no 7B The biliary structure is plate formed and *here viewed* from the edge A small defect or hole in the plate is visible because the plate is sl ghtly wavy Magni ficat on × 200 Original block size 11 × 11 × 6 5 cm

Fig 7 Same reconstruction as in *Fig 6 The plate* formed duct is here viewed from the surface It runs diagonally through the total height *of the* reconstructed block A larger defect is seen in the left side of the upper edge On account of the re fraction in the acryl block this defect is seen simul taneously through the upper surface and through the anterior left surface of the block

Fig 8 Reconstruction from b opsy no 6 From the upper frontal corner of the block an *artery (grey)* runs downwards as seen through the right frontal surface of the block Behind this artery ductal structures (black) are arranged in two separate *parallel planes A hint* of the network arrangement is apparent to the right of the artery Magnification × 200 Original block size 11 × 11 × 8 5 cm.

Fig 9 The block in Fig 8 has been turned 180° *through its vertical axis.* The network arrangement is now clearly visible In the centre of the upper *surface of the* block a plate formed segment of the network is seen This is also the case to the right in the upper surface of the block

Fig 10 Reconstruction from biopsy no 5A A complex of arteries (grey) runs vertically down *through* the centre of the block They are sur rounded by an incomplete cylinder or network of bile *ducts (black)* In the upper part of the left frontal surface of the block a plate formed segment *of the network* is seen Magnification × 200 Orig inal block size 12 × 12 × 9 5 cm

Fig 11 The block in Fig 10 has been turned up *side down* The complex of arteries is now more clearly seen The network of bile duc s is evident Behind the arteries to the left a plate formed ductal segment is seen

Regarding the distribution of q, a higher percentage of very oblong profiles (i e low q value) was found in IPCD compared to CHF (Fig 2) But these average distributions are the result of a quite pronounced spread among the individual cases as illustrated in Fig 3 The wide spread in IPCD has previously been discussed (*Jorgensen* 1973 b) The spread in CHF is less pronounced The difference in the degree of spread between the two materials may—at least in part—be referred to the number of profiles measured from each case in CHF 200 profiles and in IPCD 100 profiles The two materials share in common the error of sampling as we have no guarantee that biliary lesions in different parts of the liver are identical As can be seen from Fig 3, as judged from the materials presented here it is in many cases impossible to differentiate between the two diseases from a stereological investigation of the ductal structures It is impossible to say whether the difference in Fig 2 is accidentally or reflects a systematical difference It is conceivable that the average dimensions of the ductal plates are larger in IPCD than in CHF, or that the ductal mixture in CHF consists of a higher percentage of tubular ducts in addition to the flattened plates than is the case in IPCD But it can be concluded from the stereological investigation that the ductal lesion is fundamentally identical in the two diseases insofar as it consists of a very characteristic mixture of different ductal shapes

D Changes in the Hepatic Anatomy with Age

It is not known whether hepatic anatomy in CHF changes with age or whether it remains constant There is probably an increasing fibrosis (*Albukerk & Duffy* 1971) (*Blyth & Ockenden* 1971) and this has been related to the appearance of portal hypertension But neither the fibrosis nor the vascular system has been submitted to quantitative studies With respect to the biliary structures it is the author's impression from cytological and histological evidence that a degenerative process is going on as was the case in IPCD (*Jorgensen* 1971 b) This does not necessarily imply a quantitatively significant break down of the ductal structures If such a degradation chiefly affects the plate formed ducts the result would be a change in q distribution A grouping of the 10 specimens with respect to age of the patient at the time of biopsy did not reveal any systematic difference with respect to q distribution, neither did the biopsies from the two patients from whom material was available at intervals of several years A proportional degradation of plate formed and tubular ducts would not change the distribution of axial ratios but would result in a reduction of the number of ductal elements This may be the case but can only be proved by means of quantification In conclusion we have at present no proof of significant changes in ductal anatomy with age

L Pathogenesis

Several hypotheses regarding the pathogenesis of the ductal lesion have been presented in the literature *Norris & Tyson* 1947 suggested an abnormal extension of the normal process of degeneration segmentation and resorption of a superfluous number of provisional ducts which might or might not be cystic dilated *Landing & Wells* 1970 suggested cystic enlargement of normal ductules in normal arrangement as explanation of the pathological findings and did not include 'overproduction of ductal elements in their theory Both hypotheses tried to draw parallels between the renal and hepatic processes

It is difficult to explain why a secondary 'cystic dilatation of bile ducts should predominantly take place in one direction resulting in *flattened* structures It is more reasonable to consider this geometrical shape primary and related to a failure in the embryological development of the biliary system (*Jorgensen* 1972) The designation the ductal plate malformation has been proposed for this disorder A secondary change perceived as dilatation may take place in this primary morphology It is very variable but usually modest Only in rare cases does it

focally result in macroscopic cysts Such a case was included in the material published on IPCD (*Jorgensen* 1973 b), and the author is aware of one case of CHF which at autopsy displayed macroscopic cysts in the liver (*Klatsk n* 1973)

The author is greatly indebted to colleagues abroad, who have kindly provided the material for this investigation My gratitude is expressed to Professor G Klatskin, Yale University, U S A

[illegible]

Holland (cases 3 + 5) and Dr *F B Bronkhorst*, Arnhem Holland (cases 1 + 2)

This study has been supported by a grant from *Kong Christian den Tiendes Fond*

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THYMUS DEPENDENCY OF PERIARTERITIS NODOSA IN DOCA AND SALT TREATED MICE

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A marked degree of Periarteritis Nodosa was found in the kidneys, with fewer lesions in heart and pancreas, in unilaterally nephrectomized haired NMRI mice treated with desoxy corticosterone acetate (DOCA) and 1 per cent saline as drinking water. Similarly treated Nude littermates with congenital aplasia of the thymus failed to develop vascular lesions.

It has recently been shown that it is possible to immunize rats against their own arteriolar wall by increasing the intravascular pressure with repeated high doses of Angiotensin II (10). It was possible to transfer this immunized state with washed thoracic duct cells (10). Transfer of a similar immunological state was found after injection of washed thoracic duct cells from renal hypertensive rats to normotensive syngenic recipients (15).

The aim of the present work has been to study if this immunological state is thymus dependent. Nude mice, homozygous for the mutation nude (nu/nu) (6), seemed suitable for this purpose as they lack recognizable thymus tissue and seemed to be depleted of thymus derived T lymphocytes by both functional (7, 13, 16) and histological (3, 9, 11) criteria.

Received 25.viii.73 Accepted 25.viii.73

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MATERIAL

Animals. Mice (NMRI) carrying the mutant nude (nu/nu) and their normally haired littermates (+/?) were studied. They were kept under clean conditions and fed sterilized mouse pellets. Both sexes were used.

METHODS

4–6 weeks old mice were unilaterally nephrectomized. Commencing one week later they received 1 per cent saline as drinking water and 6 mg DOCA (Percorten® microcrystalline Ciba) subcutaneously every week. Untreated animals served as controls. After 13 weeks treatment the animals were killed with ether and the heart, kidney and pancreas were immediately fixed in 4 per cent formalin and embedded in paraffin. 5 micron thick sections were cut and stained with Van Gieson Hansen (VGH), Haematoxylin Eosin (HE) and the Periodic acid Schiff (PAS) stain. The cellular reaction around the interlobar and the interlobular/arcuate arteries of the kidney was graded from one + to three +, following a photographed scale with representative arteries. The arteries were identified on the basis of their size and topographic location in the kidney as described by *Fourman & Moffat* (1970). 30–40 serial sections of each organ were studied. The heart weight was measured and expressed as per cent of the body weight. The mediastinum was carefully dissected post mortem in all nude mice to establish absence of the thymus. No thymus was found.

TABLE 1 *Survey of the Experimental Results*

a interlobaris		a interlobularis/arcuata	
Nude	Haired	Nude	Haired
N 1 +	H 1 +++	N 1 +	H 1 ++
N 2 +	H 2 +++	N 2 +	H 2 +++
N 3 +	H 3 +++	N 3 +	H 3 +++
N 4 +	H 4 +	N 4 +	H 4 +++
N 6 +	H 5 +++	N 6 +	H 5 ++
N 7 +	H 6 ++	N 7 +	H 6 ++
N 8 +	H 7 +++	N 8 +	H 7 ++
N 10 +	H 8 +	N 9 ++	H 8 ++
N 11 +	H 9 ++	N 10 ++	H 9 ++
N 12 +	H 10 +	N 11 +	H 10 ++
N 22 +	H 11 +++	N 12 +	H 11 +++
N 25 +	H 12 +++	N 13 +	H 12 +++
N 30 +	H 13 ++	N 22 +	H 13 +++
	H 14 +++	N 25 +	H 14 +++
	H 21 +	N 30 +	H 21 +
	H 23 ++		H 23 ++
	H 25 +++		H 25 +++
	H 30 +		H 30 +++

Table 1 shows the marked difference between the cellular reaction around both the interlobar and the arcuate/interlobular arteries in haired and nude mice unilaterally nephrectomized and treated with DOCA and salt for 13 weeks

RESULTS

Group I Untreated control animals 6 haired and 5 nude mice were killed when 5-6 months old

Microscopic Investigation

The kidney the normal coat of connective tissue around the interlobar artery, continuing in the connective tissue of the pelvis was found to include few mononuclear cells and few granulocytes Around the interlobular and the arcuate arteries were found sparse and loose connective tissue and a slight cellular infiltration consisting of granulocytes fibroblasts and mononuclear cells presumably lymphocytes and monocytes, not exceeding a 1+ reaction This infiltration was of equal degree in haired and nude mice

The heart no signs of vascular disease Few mononuclear cells and sparse loose connective tissue were observed in the adventitia The relative heart weight was 0 48 per cent ± 0 04 (SD) in nude and 0 37 per cent ± 0 06 (SD) in haired mice this difference being significant ($p < 0\ 005$)

The pancreas no signs of vascular disease

Group II 17 *nude* mice, unilaterally nephrectomized and treated with DOCA and salt for 13 weeks 1 animal died during the nephrectomy and 1 after 10 weeks of treatment 15 animals were killed after 13 weeks

Macroscopic Investigation

The kidney was enlarged and the colour was pale with a slight granulation of the surface

The heart was increased in size due to a hypertrophy No infarcts or change in the colour The relative heart weight was 0 57 per cent ± 0 06 (SD) significantly greater than that of the nude control mice of group I ($p = 0\ 005$)

The pancreas was normal in all animals

Microscopic Investigation

The kidney the arteries were normal (Fig 1 and 2) An increase of the number of cells around the vessels up to a 2+ reaction was found in only 2 out of 15 animals (Table 1) The cells consisted mostly of mononuclear

cells with a morphology like lymphocytes or monocytes, some plasma cells, granulocytes and fibroblasts were present also There was no increase in the amounts of PAS positive material in the vascular walls of the arcuate and the interlobular arteries and no increase in the quantity of the surrounding connective tissue There was neither dilatation of the tubules, hyaline casts nor glomerular lesions

The heart in only 1 mouse an increase in the amounts of PAS positive material was found in the media of the arteries

The pancreas there was no vascular disease

Group III 19 *haired* mice Unilateral nephrectomized and treated with DOCA and salt for 13 weeks 1 died during the nephrectomy

Macroscopic Investigation

The kidney was enlarged, the surface granular and its colour turned from red brown to pale grey

The heart the mean relative heart weight was 0 59 per cent ± 0 12 (SD), significantly greater than that of the untreated haired mice ($p < 0\ 001$) and similar to the treated nude mice in group II ($p > 0\ 5$)

The pancreas no pathological changes

Microscopic Investigation

The kidney 17 of 18 animals had pathological cellular infiltrations around the arteries (Table 1) The *interlobar artery* of 9 of 18 animals had a 3+ cellular reaction, consisting mostly of mononuclear cells and some plasma cells granulocytes and fibroblasts (Fig 3) 4 animals showed a 2+ and 5 a 1+ cellular reaction 7 mice had increased amounts of connective tissue fibrils lying eccentrically in a great nodule on one side of the interlobar artery and in close contact with the wall (Fig 3) In 5 of these, fibrinoid necrosis was found in parts of the wall, involving all layers of the wall and in some places leucocytes infiltrated the media of the vessel while other leucocytes adhered to the endothelium on the luminal side of the vessel (Fig 4) In such places many intravascular red blood cells and granulocytes in adventitia indicated an acute reaction In 9 out of 18 mice the *arcuate/interlobular arteries* showed a 3+ cellular reaction (Table 1), (Fig 5) In a longitudinal section it was seen that the cellular reaction was present along the whole artery tree from the interlobar to the interlobular artery inclusive, but only once around an afferent artery In only 1 mouse (no 3) an increase in the amounts of PAS positive material was found in the media of an interlobular artery and an increase in the amounts of periarterial connective tissue Dilated tubules, hyaline casts and glomerular lesions consisting of partly hyalinized tufts were observed in 16 of 18 animals

The heart lesions of the blood vessels were observed in 8 of 18 animals consisting of fibrinoid necrosis of parts of the wall and periarteritis, consisting of lymphocytes, a few plasma cells, polymorphonuclear cells and fibroblasts

The pancreas in 6 animals an increase of PAS positivity of the vascular walls was observed and in one a strong cellular reaction around the arteries and total hyalinization of the arterioles

DISCUSSION

The present investigation shows a marked monocellular infiltration around the interlobar, the arcuate and the interlobular arteries of the kidney in unilaterally nephrectomized *haired* mice treated with DOCA and salt for 13 weeks *Ebihara & Mart* (1971) have shown that mice treated in this way develop hypertension The pathological picture resembles that described as Periarteritis Nodosa (PN) in experimental animals (1 8) as well as in human beings (1 2) The cellular reaction was much like that observed around damaged mesenterial arterioles in acute Angiotensin II hypertensive rats (10 15), although more marked which could be due to the longer duration of the experiment It was typical that despite a very marked perivascular cellular infiltration and in some







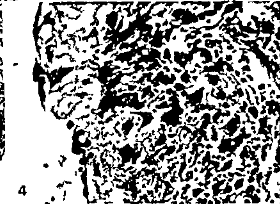




Fig 1 The interlobar artery in a *nude* mouse Treated for 13 weeks with DOCA and salt No increased cellular reaction and no media changes Magnification 70 × PAS staining

Fig 2 The interlobular artery of a *nude* mouse Treated for 13 weeks with DOCA and salt No increased cellular reaction (1 +) and no media changes Magnification 140 × PAS staining

Fig 3 The interlobar artery of a *haired* mouse Treated for 13 weeks with DOCA and salt 3 + cellular reaction nodule of connective tissue eccentrically around the vessel and fibrinoid necrosis of parts of the wall Magnification 70 × PAS staining

Fig 4 The interlobar artery of a *haired* mouse Treated for 13 weeks with DOCA and salt 3 + cellular reaction of the acute type with many blood cells in adventitia and leucocytes situated in the media Magnification 350 × VGH staining

Fig 5 The [illegible]

Magnification 140 × PAS staining

cells with a morphology like lymphocytes or monocytes some plasma cells granulocytes and fibroblasts were present also There was no increase in the amounts of PAS positive material in the vascular walls of the arcuate and the interlobular arteries, and no increase in the quantity of the surrounding connective tissue There was neither dilatation of the tubules, hyaline casts nor glomerular lesions.

The heart in only 1 mouse an increase in the amounts of PAS positive material was found in the media of the arteries

The pancreas there was no vascular disease.

Group III 19 *haired* mice Unilateral nephrectomized and treated with DOCA and salt for 13 weeks. 1 died during the nephrectomy

Macroscopic Investigation

The kidney was enlarged, the surface granular and its colour turned from red brown to pale grey

The heart the mean relative heart weight was 0.59 per cent ± 0.12 (SD), significantly greater than that of the untreated haired mice ($p < 0.001$) and similar to the treated nude mice in group II ($p > 0.5$)

The pancreas no pathological changes

Microscopic Investigation

The kidney 17 of 18 animals had pathological cellular infiltrations around the arteries Table 1 The *interlobar artery* of 9 of 18 animals had a 3+ cellular reaction consisting mostly of mononuclear cells and some plasma cells granulocytes and fibroblasts Fig 3 ? animals showed a 2+ and 5 a 1+ cellular reaction. 7 mice had increased amounts of connective tissue fibrils lying eccentrically in a great nodule on one side of the interlobar artery and in close contact with the wall Fig 3 In 5 of these fibrinoid necrosis was found in parts of the wall involving all layers of the wall and in some places leucocytes infiltrated the media of the vessel, while other leucocytes adhered to the endothelium on the luminal side of the vessel (Fig 4) In such places many intravascular red blood cells and granulocytes in indicated an acute reaction. In 9 out of mice the *arcuate and interlobular arteries* a 3+ cellular reaction (Table 1), (Fig 3) In a longitudinal section it was seen that cellular reaction was present along the whole artery tree from the interlobar to the interlobular artery inclusive but only c ... an afferent artery In only 1 mouse (no 3 an increase in the amounts of PAS material was found in the media of an interlobular artery and an increase in the amount of periarterial connective tissue Dilated tubules, hyaline casts and glomerular lesions consisting of partly hyalinized tufts were observed in 16 of 18 animals.

The heart lesions of the blood vessels were observed in 8 of 18 animals, consisting of fibrinoid necrosis of parts of the wall and periarteritis consisting of lymphocytes, a few plasma cells, polymorphonuclear cells and fibroblasts

The pancreas in 6 animals an increase of PAS positivity of the vascular walls was observed and in one a strong cellular reaction around the arteries and focal hyalinization of the arterioles.

DISCUSSION

The present investigation shows a marked monocellular infiltration around the interlobar the arcuate and the interlobular arteries of the kidney in unilaterally nephrectomized *haired* mice treated with DOCA and salt for 13 weeks. *Ebihara* & *Mc* (1971) have shown that mice treated in this way develop hypertension. The pathological picture resembles that described as Periarteritis Nodosa (PN) in experimental animals 1 8) as well as in human beings (1 2) The cellular reaction was much like that observed around damaged mesenterial arteries in acute Angiotensin II hypertensive rats (10 15) although more marked which could be due to the longer duration of the experiment. It was typical that despite a very marked perivascular cellular infiltration and in some

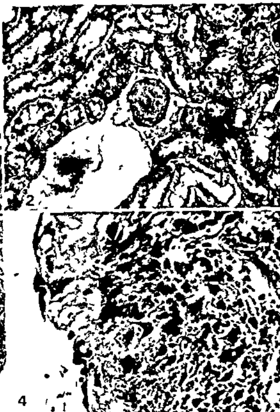



Fig 1 The interlobar artery in a *nude* mouse Treated for 13 weeks with DOCA and salt No increased cellular reaction and no media changes Magnification 70 × DAS staining

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Fig 3 The interlobar artery of a *haired* mouse Treated for 13 weeks with DOCA and salt 3+ cellular reaction, nodule of connective tissue eccentrically around the vessel and fibrinoid necrosis of parts of the wall Magnification 70 × PAS staining

Fig 4 The interlobar artery of a *haired* mouse Treated for 13 weeks with DOCA and salt 3+ cellular reaction of the acute type with many blood cells in adventitia, and leucocytes situated in the media Magnification 350 × VGH staining

Fig 5 The interlobular artery of a *haired* mouse Treated for 13 weeks with DOCA and salt 3+ cellular reaction and hypertrophy of the media Magnification 140 × PAS staining

cases hypertrophic media, fibrinoid necrosis of the wall of the interlobar artery was only found in 5 haired mice Further, dilated tubules, hyaline casts, hyalinization of parts of the tubules and infarcts in the kidneys were present

None of these lesions were found in kidney, heart or pancreas from similarly treated *nude* mice It is known that nude mice suffer from several deficiencies and that the number of circulating lymphocytes is decreased (12) The only difference between the nude DOCA treated and the nude control animals was a significant increase in the relative heart weight, giving evidence that the blood pressure had been elevated in the DOCA treated group (14) The difference in vascular lesions in haired and nude mice may be caused by the difference in T cells, which are believed to be the effector cells of delayed type immune responses

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The author is grateful to Dr *M H Claesson*, the University Institute of Anatomy, Dept A for the experimental animals and to *Miss Lisbeth Olsen* for technical assistance This work was supported by grants from the *Danish Medical Research Council* and the *Danish Heart Association* The Percorten® was kindly supplied by Ciba Geigy, Copenhagen

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ON THE PRESENCE WITHIN TUMOURS OF CLONES THAT DIFFER IN SENSITIVITY TO CYTOSTATIC DRUGS

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An *in vitro* test model was used to study the heterogeneity of tumours with respect to the sensitivity to cytostatic drugs Methylcholanthrene induced sarcomas in mice were used The effect of the drugs was determined as the reduction of the incorporation of H^3 thymidine into drug treated cells compared with control cells To test the heterogeneity of the tumours these were cut into four equal parts and each part was tested separately In some of the tumours, the quarters differed significantly in their sensitivity to the same cytostatic treatment, in others no such differences could be found Both primary and serially transplanted tumours were studied The present method revealed no differences in heterogeneity of the tumours in these two groups The most likely explanation of these results is that the tumours are built up by different clones with different sensitivity to cytostatic drugs and that different parts of the tumours are different with respect to the distribution of clones

The possibility of using *in vitro* tests for the prediction of sensitivity of tumours to cytostatic drugs has been much explored, but no method has as yet been described which can be used as a reasonably accurate predictive test We have been studying an *in vitro* method which estimates the sensitivity of a sample of tumour cells to three cytostatic drugs melphalan, vinblastine sulphate and cytosine arabinoside This method has been evolved on methylcholanthrene induced mouse sarcomas An earlier paper (1) showed that different tumours respond differently in this test and that the variation in response was more pronounced if a number of primary sarcomas were studied than if a group of serially transplanted tumours were compared Using tumours that had been transplanted for ten generations, however, we still noted a residual heterogeneity between the daughter tumours with respect to *in vitro* sensitivity to the drugs This could be due to biological differences, but it could also mirror experimental sources of variation, as the tumours were studied in separate experiments

In this paper, the model is further explored with a view to investigating whether differences, with respect to *in vitro* response, can be demonstrated *within* tumours, the existence of such a heterogeneity could indicate within a sarcoma the presence of clones that differ in sensitivity to cytostatic drugs Furthermore, such a finding would eliminate the possibility that technical variations cause the recorded differences, as the various parts are analysed simultaneously

MATERIAL AND METHODS

Details of the methods are given in a previous paper (1) Briefly subcutaneous sarcomas

Received 2 vii 73 Accepted 2 vii 73

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induced in C_{57} black female mice using 3 methylcholanthrene Primary tumours appeared after approx three months and were then used for testing In one experiment, a sarcoma was serially transplanted for ten generations by subcutaneous injection of a tumour cell suspension containing 5×10^{6} cells After ten passages, the final tumour was transplanted in the same way to six different recipients Three weeks later, tumours had developed These were used for the test Parallel to the investigation of these six serially transplanted tumours, six primary sarcomas were studied in tests running completely parallel In an earlier series, six primary sarcomas had been tested Thus the final material consists of twelve primary tumours and six tumours that had been transplanted serially

At the test, each tumour was divided into four roughly similar portions From each quarter, a cel suspension was prepared as described previously The cell suspensions were incubated for three hour in the presence of cytostatic drugs or with culture medium alone Tritiated thymidine was then added and incubation continued for a further hour Thymidine incorporation into DNA was determined with the aid of liquid scintillation The DNA content of each sample was also determined and DNA synthesis was expressed as isotope incorporation per DNA units Details of media used, drug concentrations, radioactivity added, DNA determination, and scintillation methods are given in (1)

In order to get near normally distributed variates suitable for statistical analysis and especially for variance analysis, the following expression was used (4)

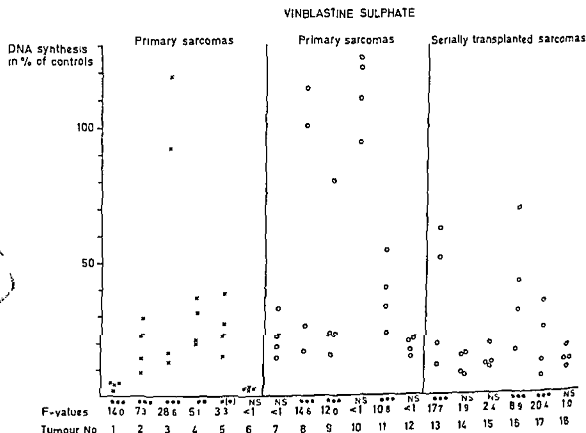


Fig 1 A

Fig 1 Diagram illustrating variability between quarters of 18 mouse sarcomas with respect to sensitivity for vinblastine sulphate (A) melphalan (B), and cytosine arabinoside (C) Each dot marks the thymidine uptake per DNA units in the presence of the drugs expressed in per cent of uptake in control cultures Variance ratios (F values) with significance levels marked are given for each tumour, estimating the variability between quarters (cf Table 1)
NS = non significant, * 0 05 > P > 0 02 *(*) 0 02 > P > 0 01, ** 0 01 > P > 0 001, *** P < 0 001
Primary sarcoma No 7–12 were studied simultaneously with the serially transplanted sarcomas No 13–18

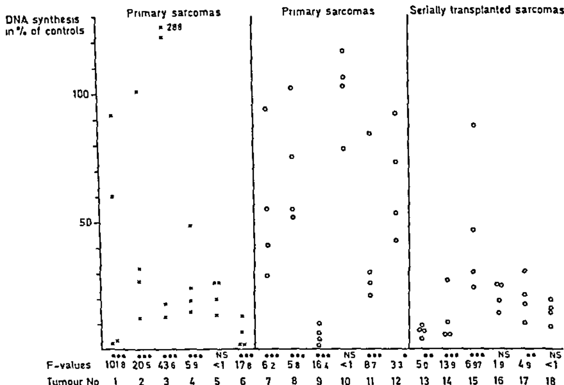


Fig 1 B

$$a = 100 \times \log_{10} \frac{\text{c p m} \times 10^4}{(\text{AES}) \times (\text{DNA})}$$

where c p m is the number of counts registered during 10 minutes (AES) is the c p m registered with automatic external standardization and (DNA) is the amount of DNA in the sample in arbitrary units Effects of a drug are expressed as the difference between the mean a value of three control tubes and the mean a value of three tubes incubated with the drug

RESULTS

Fig 1 describes the results DNA synthesis in the presence of drugs is here expressed as the percentage of control levels (= 100 per cent) Thus, at 0, the drug has extinguished all DNA synthesis *Plotting of the data in this* way permits a direct estimate of the degree of effect It will result, however in an apparent reduction of dispersion at low values and an apparent increase of dispersion at high values For each tumour studied the values for each quarter are marked separately

For a certain drug and a certain tumour, 24 incubations were performed three control tubes and three drug tubes for each quarter of the tumour These 24 determinations show a variation caused by the following known sources a) technical error (i e variation between identically performed triplet tubes), b) presence or absence of drug, c) tumour quarter The statistical interaction between the two latter sources of variation estimates the variation in sensitivity to the drug in question in the four quarters An analysis of variance can be performed to estimate this interaction, Table 1 exemplifies such an *analysis If the variance ratio (F value)* obtained for interaction is significant, it means that the four quarters do not respond in a similar way in the *in vitro* test F-values and significance levels are marked in Fig 1 for

CYTOSINE ARABINOSIDE

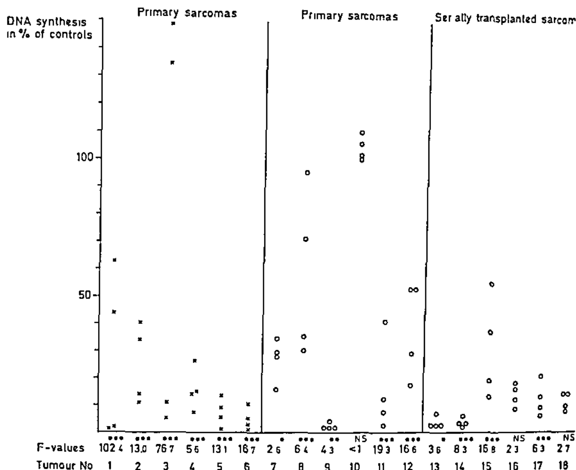


Fig 1 C

all twelve primary tumours and for the six serially transplanted tumours It should be remembered that six of the primary tumours are tested in experiments run completely parallel to those testing the serially transplanted sarcomas

Fig 1 gives the impression that the scattering of the effect values for primary tumour quarters is larger than that for serially transplanted tumour quarters, but differences between individual tumours are large In one tumour, for instance (No 1), two of the studied quarters showed a very strong reactivity to melphalan the third quarter was intermediate (approx 60 per cent of control value), and the final quarter appeared insensitive (92 per cent of control value) In many other tumours, all four quarters showed very similar response

In an effort to evaluate statistically whether the degree of heterogeneity, as estimated in the present way, was actually of different magnitude in different tumours and whether there were any statistically significant differences in result between primary tumours and serially transplanted tumours, an analysis of variance was performed (see Table 2) As no difference between the two groups of primary tumours was found (those tested earlier than the serially transplanted tumours and those tested simultaneously with them) the two groups were added in the statistical analysis The Table shows the result of an analysis of the contributions to the total interaction be-

TABLE 1 *Example of Analysis of Variance Studying Variability in Response to Melphalan between Quarters of a Mouse Sarcoma*

Source of variation	n	$\Sigma (X-\bar{X})^2$	variance	F
Total	23	213519 8667		
Between quarters	3	124264 2413		
Between controls and drug	1	49741 5199		
Error	16	1950 68	121 92[a]	
Interaction	3	37563 4252	12521 14	101 80***

[a] general error variance, common to the whole material, is 123 at 221 d f
*** $P < 0\,001$

tween the sources of variation "quarters" and "effect of drug" The influence of "groups" (i e primary tumours *contra* serially transplanted tumours) and the influence of "tumours" (strictly speaking also of host animal and experimental situation) can be compared with the interaction within tumours Table 2 makes it obvious that the source of variation "tumour" is of great importance and that the influence of "group" is not significant compared with that of "tumour'

If thus analysed, it is impossible to demonstrate a significantly greater homogeneity in serially transplanted tumours than in primary ones

DISCUSSION

From the aspect of *in vitro* sensitivity to cytostatic drugs, methylcholanthrene induced mouse sarcomas are thus heterogeneous to the extent that, if divided into quarters, easily demonstrated differences between the four pieces can often be found, even varying from complete insensitivity to almost complete sensitivity to the drugs in question Such findings cannot be explained by a spatial mixing of different cell clones—the relatively large pieces studied in this investigation should then give representative samples of the average cell composition of the tumour and be almost identical Instead, relatively large regions with different qualities with respect to drug sensitivity must be supposed to exist Rather similar findings obtained in studies of the cytogenetic properties of tumour cells have been published It is well known that many tumours contain a number of different stem-lines with different modal chromosomal numbers *Levan* (quoted from 3) found within a localized part of a primary

TABLE 2 *Variance Analysis of Contributions to the Interaction between 'Quarters of Tumours" and 'Effect of Drug'*

		Vinblastine sulphate		Melphalan		Cytosine arabinoside	
Source of variation	df	variance	F	variance	F	variance	F
Between groups (primary and serially)	1	2352	<1NS	10559	1 13NS	6551	<1NS
Between tumours within groups	16	8569	8 34***	9322	5 31***	8435	3 78**
Within tumours (− between quarters)	54	1028		1757		2231	

NS non significant
** $0\,01 > P > 0\,001$
*** $P < 0\,001$

Rous rat sarcoma a hypodiploid chromosome number, the remaining part of the tumour having a diploid number *Mittelman* (3) studied eleven primary rat sarcomas cytogenetically In two, he found different stem-lines *in two different parts of the tumour* Also these findings demonstrate that large regions of different cell clones can exist within one and the same tumour

The present experiment showed that the degree of heterogeneity varied considerably in different primary sarcomas some appeared nearly homogeneous, others showed a marked degree of heterogeneity This is to be expected if large regions with different properties exist in a tumour cut arbitrarily into four pieces This great variation in tumours, with respect to heterogeneity, made it impossible to demonstrate an effect of serial transplantation on the degree of heterogeneity In a later paper (2), this phenomenon shall be further analysed

Because there is often a great degree of heterogeneity within a mouse sarcoma, the predictive value of a test on a biopsy from such a tumour would be very low—even if there was a test method which *per se* gave a good correlate to the *in vivo* reaction of the cells to the cytostatic drug under study If the same is true of human malignant tumours —as can reasonably be supposed—this problem is of great importance, as all *in vitro* methods are based on estimates of the sensitivity *of samples removed from* a *large and* often diffusely distributed tumour mass

The cost of this investigation was defrayed by grants from research found of *Medical Faculty* and the *John and Augusta Persson Foundation*

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Acta path microbiol scand Section A *82*, 41–47, 1974

CELL CLONES WITH DIFFERENT SENSITIVITY TO CYTOSTATIC DRUGS IN METHYLCHOLANTHRENE-INDUCED MOUSE SARCOMAS

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One primary and one serially transplanted methylcholanthrene induced mouse sarcoma was stud ed for the existence of cell clones that differ in their sensitivity to cytostatic drugs Two transplantation methods were compared One method was to graft a small sarcoma piece subcutaneously a second method was to inject a small volume of a cell suspension prepared from the sarcoma After a growth period the transplanted tumours were tested for cytostatic sens tivity *in vitro* Reduction in H^3 TdR incorporation measured the effect of the cytostatic drug added The tumours obtained in the recipient mice differed in sensitivity *in vitro* Tumours obtained by grafting material from the serially transplanted sarcoma did not differ in sensitivity more than those obtained by injection transplantation Tumours obtained by grafting pieces of the primary sarcoma showed a significantly more marked degree of diversity than the tumours obtained by injection transplantation of that sarcoma

In a previous paper, the present authors described an *in vitro* method for the study of the sensitivity for cytostatic drugs of cell suspensions prepared from methylcholanthrene induced mouse sarcoma (1) In another investigation (2) we used this method to study the homogeneity of such sarcomas The problem was investigated by comparing the reaction of four different parts of a tumour to three cytostatic drugs It revealed that different tumours appeared differently heterogeneous Compared with primary induced sarcomas, those obtained after serial transplantation for ten generations showed a tendency towards less heterogeneity but owing to the great inter tumour variation, this finding could be random

Received 15 viii 73 Accepted 18 ix 73

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The present investigation used another method to further investigate the heterogeneity of primary and serially transplanted sarcomas

MATERIAL AND METHODS

Sarcomas were induced by subcutaneous injection of 3 methylcholanthrene (1) Syngenic female C57 black mice were used After about three months, tumours developed One primary sarcoma and one serially transplanted for ten generations were used for this study

Each tumour with an approx diameter of 10–15 mm was dissected free from non tumorous tissue and cut into pieces weighing appr 25 mg (23–26 mg) The pieces from one tumour were mixed, and 18 pieces from the primary tumour and 14 from the serially transplanted tumour were selected at random and transplanted subcutaneously to nine and seven recipients, respectively, each recipient thus had two grafts one in each flank The remaining pieces of the two tumours were brought into cell

suspensions These were then injected subcutaneously, 5 million cells in a volume of 0 1 ml of Parker 199 (SBL, Stockholm), in each flank of the recipients The primary tumour was transferred to six recipients, and the serially transplanted tumour to ten

All tumour cell suspensions thus injected gave rise to tumours within 14 days Graft transplants produced tumours after 3–4 weeks Within each group, tumours were used which developed after approx equally long growth periods and which were approx uniform in size From these tumours, cell suspensions were prepared and the *in vitro* sensitivity to vinblastine sulphate, melphalan, and cytosine arabinoside was determined, see footnote 1 Details of the *in vitro* method are given elsewhere (1) Briefly, cell suspensions were prepared and incubated in a medium with or without the drugs for three hours Tritiated thymidine was then added, and the incubation continued for another hour Thymidine incorporation into DNA was then determined with liquid scintillation and the DNA content of the sample was determined spectrometrically with an indole method To obtain near normally distributed variates (cf 3) the thymidine uptake in a certain sample was expressed

$$a = 100 \times \log_{10} \frac{\text{c p m} \times 10^4}{(\text{AES}) \times (\text{DNA})}$$

Vinblastine sulphate (Velbe®, Lilly, Indianapolis) was used in a final concentration of 100 µg/ml, melphalan (Alkeran®, Burroughs and Wellcome, London) in a concentration of 200 µg/ml, and cytosine arabinoside (Sigma, S t Louis) in a concentration of 250 µg/ml

where (AES) is the c p m registered with external standardization, and (DNA) is the amount of DNA in the sample expressed in arbitrary units

The effect of a cytostatic drug on a cell suspension is evaluated as the difference of the mean a value for three controls where the cells are incubated without the cytostatic drug added, and the mean a value for three samples incubated with the drug In a few instances, means were calculated from two instead of three parallel incubations

RESULTS

The histological picture of the daughter tumours obtained from the primary tumour was very similar irrespective of transplantation method Also the daughter tumours from the serially transplanted sarcomas were uniform, but were more anaplastic in appearance

The *in vitro* sensitivity of each daughter tumour to vinblastine sulphate, melphalan and cytosine arabinoside was tested, Figures 1 A C give the results Each animal is represented by two circles, one for each of the two tumours carried by the same animal The effects of the cytostatic drugs are expressed as remaining DNA synthesis in per cent of untreated controls The percentages were calculated using antilog a-values Presenting the data in this way gives a better visualization

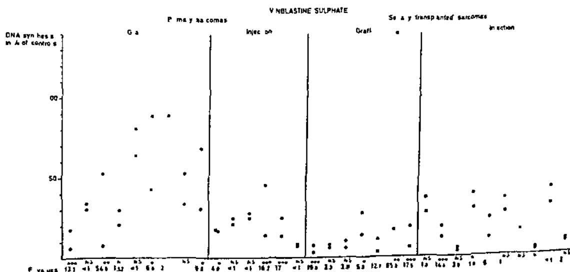


Fig 1 A

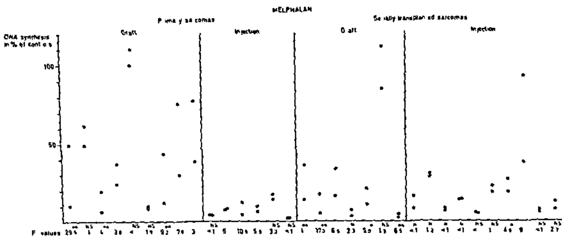


Fig 1 B

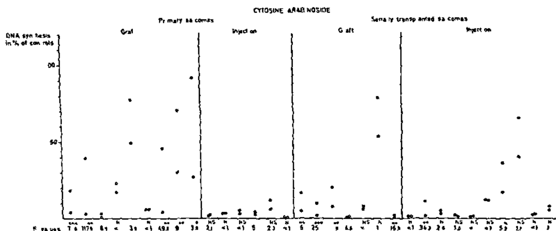


Fig 1 C

Fig 1 Diagram illustrating variability between daughter tumours with respect to sensitivity for vinblastine sulphate (A), melphalan (B) and cytosine arabinoside (C) Each circle marks the thymidine uptake per DNA units in the presence of the drugs expressed in per cent of uptake in control tubes Variance ratios (F values) obtained for each animal is given at the foot of the figure NS non significant *0 05>P>0 02, **0 01>P>0 001 ***P<0 001

of the effects than the differences in *a* values, but it also results in an apparent increased dispersion at high values and a decreased dispersion at low values The statistical analysis is therefore performed on the *a* values

In order to evaluate whether the two tumours on the same animal differ in their reaction to the drugs an analysis of variance is performed to estimate the interaction between the two sources of variation "tumours" and "drug effects" (cf 2) The variance obtained is compared with the technical error variance (i e between identically performed incubations), which in the present material is found to be 138 estimated from 479 d f (which is a reasonable estimate of the general error variance 123 based on 223 d f used in a previous report) (2) The variance ratio obtained for each animal is given at the foot of Fig 1

TABLE 1 *Analysis of Different Sources of Variation, Contributing to the Heterogeneity of Tumours Studied, Estimated as Interaction between "Tumours' and "Drug Effects" F values Give Variance Ratios*

Source of variation	d f	vinblastine sulphate		melphalan		cytosine arabino-	
		variance	F	variance	F	variance	F
Between "graft" and "injection" technique							
Tumours from primary sarcoma	1	11580	7 4*(*)	57898	15 3***	68148	6 1
Tumours from serially transpl sarcoma	1	947	$<1^{NS}$	2231	$<1^{NS}$	4337	<1`
Between "animals" within transplantation technique and tumour group	28	2987†	2 4*	3776	5 8***	11200	9
Within animals	32	1247		650		1239	
Interaction between "effect of tumour group" and "effect of transplantation method'	1	32317	10 8**	29329	7 8**	29952	$2\ 7^{NS}$

† The 'between animals" variation differs significantly for tumours obtained from the primary sarcoma and for those obtained from the serially transplanted sarcoma (variances 1561 and 4615, resp), when studied with vinblastine sulphate, but not with the other drugs A weighted variance was therefore used for the further analysis of the vinblastine sulphate, based on 24 d f (cf 4)

Fig 1 suggests that there is a greater variation in tumours obtained with the graft method than in those obtained with the injection technique Tumours obtained from the primary sarcoma also appear to be more heterogeneous than those obtained from the serially transplanted sarcoma To evaluate the contribution of the different sources of variation, an analysis (see Table 1) was performed For all three drugs, "animal' is an important source of variation within both transplantation types (graft transplantation and injection transplantation) being most pronounced for cytosine arabinoside

The effect of the used transplantation method is found to be statistically significant if material from the primary sarcoma is employed Thus, tumours obtained by graft transplantation strongly diverge in sensitivity *in vitro* for cytostatic drugs, whereas tumours obtained with the injection technique diverge little. The variance due to "between transplantation methods" is of the same approximate magnitude for all three drugs Tumours obtained from the serially transplanted sarcomas studied in the same way show that the variance due to differences in transplantation methods is even smaller than that registered for the "between animals" variation Analysis of the interaction between the two sources of variation "between tumour groups (i e tumours obtained from the primary sarcoma and tumours obtained from the serially transplanted sarcoma) and "between transplantation methods" (i e graft transplantation and injection transplantation) gives a variance value of appr the same size for all three drugs It is statistically significant for vinblastine sulphate and melphalan but does not reach statistical significance for cytosine arabinoside owing to the high 'between animals" variation for this drug

This analysis thus shows that there is a higher degree of heterogeneity between tu-

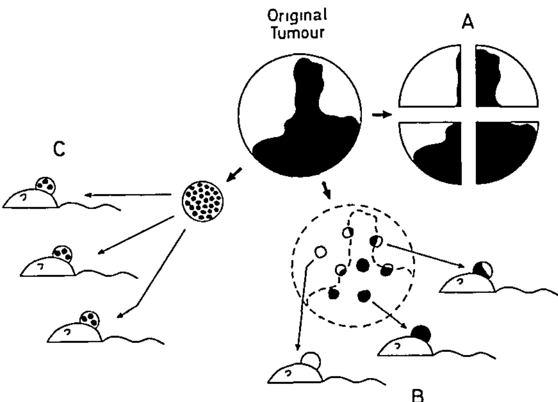


Fig 2 Principles in experiments on heterogeneity Original tumour built up by two clones, marked black and white resp A division of tumours into quarters followed by *in vitro* testing of each quarter B Transplantation of small grafts selected at random and *in vitro* testing of tumours that develop C Transplantation of homogeneous cell suspension and testing *in vitro* of tumours that develop

mours obtained from the primary sarcoma, if tested with cytostatic drugs *in vitro*, if developed from grafted pieces of sarcoma tissue than if developed from injected suspensions of sarcoma cells This difference becomes smaller and even disappears when tumours obtained from the serially transplanted sarcoma are studied

DISCUSSION

The present authors studied methylcholanthrene induced mouse sarcomas from the standpoint of heterogeneity in *in vitro* sensitivity to cytostatic drugs Two methods were used In an earlier paper (2) we described data comparing the *in vitro* sensitivity of quarters of a number of tumours, here we transplanted small pieces from a tumour to recipients and studied the *in vitro* sensitivity of the tumours that developed from the grafts Fig 2 illustrates schematically the principles of these methods For simplicity, only two clones with different sensitivity to cytostatic drugs are shown In the first method (Figure 2A), the tumours were divided into quarters and tested separately *in vitro* Some tumours were found to be heterogeneous, others were not Considered from this point of view, primary and serially transplanted tumours did not differ This might indicate that the tumours were still markedly heterogeneous even after serial transplantation for ten generations in the absence of evolutionary pressure, the tumours were permitted to grow without any drug treatment of the animals But it is also possible that the tumours had developed towards less heterogeneity during

the serial transplantation, although this could not be demonstrated with the method used Differences in heterogeneity between different kinds of tumours can be obscured, e g the quartering of the tumour can be done in many different ways with quite variable results, even if only two unevenly distributed clones are present in the tumour Thus, the intertumour variation with respect to the degree of heterogeneity will be highly influenced by the way a tumour is quartered

The present method was developed to analyse the heterogeneity of tumours further (Fig 2B) Small pieces of an original tumour were grafted to a number of animals The tumours that developed from the grafts were then tested for their sensitivity to cytostatic drugs *in vitro* If the original tumour is built up by different clones that are not intermingled, the small pieces of that tumour ought to be different in their set up of clones, consequently, tumours developing from such pieces ought to be different On the other hand, heterogeneity can develop during the further growth of the graft in the recipient mouse Selection processes and mutations might work, furthermore, local factors like nutritional effects could cause an apparent heterogeneity To evaluate the importance of such processes, parallel experiments were made with tumours obtained from injection of cell suspensions These suspensions should give a representative average of the cell composition of the tumour, and all transplanted tumours should develop from similarly composed samples (Figure 2C) If there is a heterogeneity, this could reasonably develop during tumour growth and not be due to variations in tumour cell inocula When tumours obtained from a primary sarcoma were investigated in this way, those obtained by [illegible]

tion transplantation However, a heterogeneity was present also among the latter, this is reasonably explained by factors of the nature discussed al ve

When tumou obtained from a serially transplanted sarcoma (ten generations) were used, no difference in heterogeneity could be shown among tumours developed from grafts and those from injected cell suspensions In both groups, a heterogeneity exists, but they are of the same size Again, this is reasonably due to the processes, commented on above The picture suggested by these data is In primary induced sarcoma there exists at least two, and perhaps many, different clones of cells differing in sensitivity *in vitro* to cytostatic drugs The clones are not completely intermingled, but form spatially separate tumour regions They have developed either from separate foci of carcinogenetically transformed cells, or during the further evolution of the sarcoma During serial transplantation, the tumour becomes more homogeneous, i e it is built up by fewer clones or the existing clones become strongly intermingled This will increase the chance for the transplanted pieces of the original tumour to become rather identical, thus the difference due to transplantation technique will be reduced However, the phenomenon that one clone shows a selective advantage over another also at growth in the absence of the cytostatic drugs in question indicates that these properties are coupled with other characteristics e g , rapidity of growth In a previous paper (1) it was shown that when the effects of the three cytostatic drugs were correlated, only the pair melphalan cytosine arabinoside showed a correlation This observation speaks against the explanation that variations in drug sensitivity is a mere mirror of cell multiplication rate

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The cost of this investigation was defrayed by grants from research fund of *Medical Faculty* University of Lund and the *John and Augusta Persson Foundation*

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Acta path microbiol scand Section A 82, 48–70, 1974

EFFECTS OF HOMOZYGOSITY OF THE *NUDE (NU)* GENE IN THREE INBRED STRAINS OF MICE

A Detailed Study of Mice of Three Genetic Backgrounds (BALB/c, C3H, C57/BL/6) with Congenital Absence of the Thymus (Nude Mice) at a Stage in the Gene Transfer

JORGEN RYGAARD and CARL O POVLSEN

The Pathological Anatomical Institute, Kommunehospitalet, DK-1399 Copenhagen K, Denmark

The study concerns comparisons between *nude* mice of three genetic backgrounds (BALB/c, C3H & C57/BL) after the third cycle of a gene transfer, and normal members of the respective three inbred strains The following parameters were considered body and organ weight, haematological values, differential counts of bone marrow and peripheral blood cells, serum proteins (total and fractionated), and detailed histological appearances of tissues and organs No thymus tissue was found in any *nude* mouse Lymphoid tissue in the *spleen, Peyer's patches and lymph nodes was of characteristic histological appearance* with lymphocytic depletion Additionally, marked difference in phagocytic activity in Peyer's patches was observed Such activity was meagre or absent in *nude* mice The possible relationship of this finding with low IgA levels is discussed Peripheral blood lymphocyte counts in *nudes*, irrespective of genetic background, were uniform and consistently very low It is proposed that these lymphocytes represent a stable B cell population Lymphocyte counts in the bone marrow were somewhat low in all *nudes* No quantitative differences in serum protein values could be demonstrated by the methods used Histological study de *monstrated inflammatory changes in one or more organs in* 11 of the 24 nude mice despite the fact that all animals were apparently healthy No changes were found in the normal mice The mice were reared under spf (specified pathogen free) conditions The insufficiency of current spf definitions in relation to *nude* mice is stressed *The infections were* always ascending, and frequently involved the genital tract The significance of possibly impaired IgA secretion is discussed The genital infections are considered in relation to the infertility of *nude* females

Mice with congenital absence of the thymus, *nude* mice, (*Isaacson & Cattanach* 1962, *Flanagan* 1966, *Pantelouris* 1968) are being studied the world over to ever increasing extent, especially in departments of immunology and oncology Yet further use will be found for these animals *in many different* branches of biological research in the years to come

This explosion of interest which has required the rapid availability of larger numbers of *nudes* has entailed *a rapid breeding* and consequent poor definition and description

Received 6 vii 73 Accepted 6 vii 73

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of the animals used, which are probably highly various in their genetic backgrounds. This makes it difficult to compare findings from different laboratories, and it is not possible to differentiate positively between the effects of the *nu* gene, and effects which depend on difference between the other elements of the genetic constitution.

In obviation of many of these difficulties we applied the same study methods in *nude* homozygous mice at the same stage in a gene transfer (*Snell* 1948, *Green* & *Doolittle* 1963) to three different inbred strains of mice, and in normal representatives of the three strains.

The observations in each study group stand in their own right, and, additionally, the findings in the three unadulterated strains can be used as reference or 'normal' controls for *nude* mouse studies.

This study concerns an intermediate stage of gene transfer and will be compared, subsequently, with studies in which gene transfer is complete. This will further elucidate and isolate effects dependent on the *nu* gene alone.

The study comprises macroscopic and microscopic examinations of organs and tissues, and haematological status—serum proteins, and differential counts in both peripheral blood and bone marrow. Main emphasis is laid on the constitution of lymphoid and haemopoietic tissues.

MATERIALS AND METHODS

The *nude* mice had the following three genetic backgrounds:

1) BALB/c/A/BOM$_{I}$ $(1-(\frac{1}{2})^{3})$, four females and four males
2) C3H/Tif/BOM$_{I}$ $(1-(\frac{1}{2})^{3})$, four females and four males
3) C57/BL/6J/BOM$_{I}$ $(1-(\frac{1}{2})^{3})$, four females and four males

The expression $(1-(\frac{1}{2})^{n})$ denotes the fraction of genome of the inbred strain, in that $(\frac{1}{2})^{n}$—n being the number of crosses—corresponds to the non-defined genetic component.

The reference groups comprised four males and four females from each of the three corresponding inbred strains.

All mice were between six and eight weeks old.

The mice were supplied from Gl. Bomholtgaard, DK 8680 Ry, Denmark. They were transported to the Pathological Anatomical Institute, Kommunehospitalet, Copenhagen in sterile cardboard boxes with glass wool filters. Temperature in the transport vans was thermostatically controlled.

The mice were kept for one week in a barrier screened spf room (cfr. *Rygaard* & *Friis* 1973).

Makrolon® type II cages were used, each containing 4 or 5 animals. Pellet feed was autoclaved at Gl. Bomholtgaard. Drinking water, *ad lib.*, was distilled, and 2 ppm chlorine was added. Room temperature at Gl. Bomholtgaard was 24 ± 1° C, and at the Pathological Anatomical Institute, Kommunehospitalet 27 ± 1° C. Relative humidity in both centres was 55 ± 5 per cent. A diurnal light cycle of 11 hours with 1 hour dawn and 1 hour dusk was provided at the Pathological Anatomical Institute, Kommunehospitalet. Mice were transferred to the laboratory immediately before sacrifice, which took place between 8.30 a.m. and noon.

Physical Examination, Blood Sampling and Autopsy Techniques

All the mice were apparently healthy on physical examination. Weights are given in Table 1.

The animals were lightly anaesthetized with ether, and pinned to a Styropor® board in the supine position. Anaesthesia was maintained until death.

Blood Sampling

A midline incision was made through the thoracic and abdominal skin, and the right axillary artery and vein dissected free. A pouch was constructed from the skin of the lateral thoracic wall. The axillary vessels were cut and the blood allowed to flow freely into the pouch.

All the blood was aspirated into disposible 2 ml calibrated syringes through 18 g siliconed needles. Total blood volume was measured. Two smears for differential counts were made on clean slides which were dried immediately with a Braun® thermolator. Preparations were fixed in methanol at room temperature for 5 min. and stained with May Grunwald Giemsa before microscopy.

Autopsy Technique

After exsanguination, autopsy was made. Organ weights are shown in Table 1.

Axillary and inguinal lymph nodes were dissected free.

The abdominal cavity was opened by a midline

lia were removed and studied Mesenteric lymph nodes and pancreas were eviscerated together

The thorax was opened by two parasternal cuts, which allowed free inspection of the superior mediastinum, while leaving the sternum undamaged for microscopic study

The thymus—when present—was isolated The trachea was transected high up, and heart and lungs were removed *en bloc* for fine dissection

Sternum, lumbar spine and adjacent muscles, one hind leg, one fore leg, part of the tail, and a piece of skin from the lateral abdominal wall were taken for histological study

The skin was removed from the cranium, which was opened by an occipital incision

The whole was fixed in neutral phosphate buffered formalin 4 per cent

Bone Marrow Preparations

The right femoral shaft was isolated and the medullary content was pressed out with a blunt stainless steel probe, and put into 1 5 ml of phosphate buffered saline with 6 per cent human albumin (PBS/HA) The medullary fragments were brought into suspension with a Vortex® mixer, centrifuged at 1500 rpm washed twice with PBS/HA, and resuspended in PBS/HA in a concentration of approximately 250 cells/μl Twenty μl of this suspension with two drops of PBS/HA were introduced into Shandon Cytocentrifuge®, and centrifuged on o clean microscope slides at maximum speed for 10 min Duplicate slides were made for each mouse After drying slides were fixed in methanol at room temperature, and stained with May Grunwald Giemsa

Histological Technique

The tissues mentioned were fixed in formalin, decalcified when necessary, and embedded in paraffin wax The 7 μm sections were stained with H&E van Gieson Hansen and methyl green pyronine (*Pearse* 1960)

LABORATORY INVESTIGATIONS

White Blood Cell Counts (WBC) Red Cell Counts, Haematocrit, Haemoglobin etc

Approximately 100 μl of blood was collected in DEAE coated dry microt ibes Determinations were made in the Departme of Clinical Chemistry at Sundby Hospital, Copenha en within two hours of blood sampling Routin laboratory methods were used Cells were cou d with a Coulter Counter®, Model B *Mean* (*Volume* (MCV), and Mean Cell Haemogl Concentration (MCHC), were calculated T results are given in Tables 3 and 5

Serum Proteins

The remaining blood was placed in 2 or 3 Sanz plastic tubes with no additions, and left to clot in the upright tubes for one hour at room temperature, and for the next 18 hours at + 4° C The next day, the tubes were centrifuged after extraction of the clot, and the serum was pipetted into sterile tubes of the same type, deep frozen, and stored at −20° C This serum portion was used for study of the serum proteins

Total serum protein was determined in the Department of Clinical Chemistry, Sundby Hospital, by a standard micro method Agarose electrophoresis a m Laurell was performed in the Department of Clinical Chemistry, Kommunehospitalet After staining with amidoschwartz and drying, glasses were cut with a glazier's diamond, and fractions as given in Table 4, were eluted in 50 per cent buffered methanol

Relative quantities were measured with a Beckmann® spectrophotometer at 590 nm, and the fractions were calculated from the total serum protein values The results are given in Table 4

HISTOLOGY AND CYTOLOGY

Peripheral Blood

The morphology and staining properties of red blood cells and thrombocytes were noted Two hundred white blood cells were classified as neutrophil granulocytes, eosinophil granulocytes lymphocytes or monocytes according to the criteria given below The results, shown in Table 5 are collected into two main groups granulocytes which comprises both neutrophils and eosinophils, and lymphocytes, which includes both lymphocytes and monocytes

Bone Marrow Preparations

A Jena Pictoval® microprojector was used The microscope picture was projected on to horizontal

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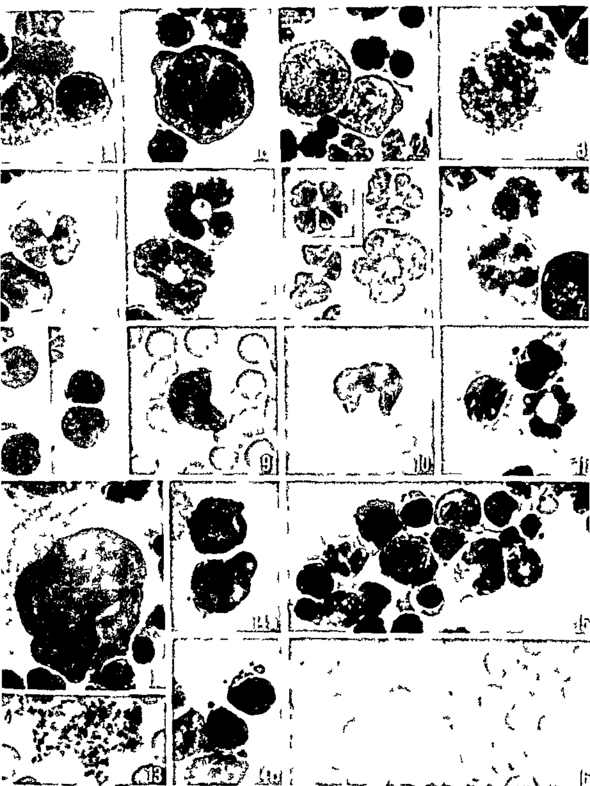
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Fig 1–16 Cells of bone marrow and peripheral blood 1 Haemocytoblast 2 Promyelocyte 3 Myelocyte, *neutrophil* 4 Metamyelocyte, neutrophil segmented line 5 Metamyelocyte, neutrophil, annular line 6 Granulocyte, neutrophil 7 Metamyelocyte, eosinophil 8 Lymphocyte, 'small' 9 Lymphocyte, 'large 10 Monocyte 11 Plasma cell 12 Megakaryocyte 13 Thrombocyte 14 Basophil erythroblast 15 Intermediate and late normoblasts 16 Erythrocytes



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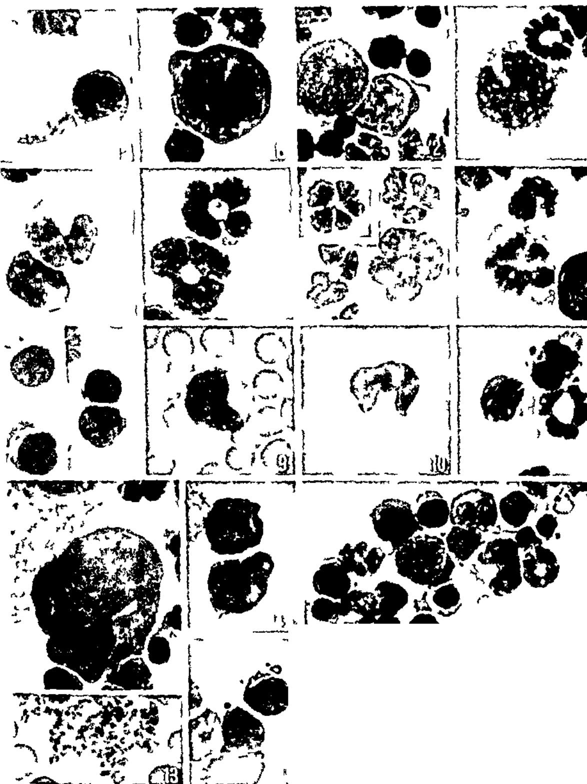
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Classification of Peripheral Blood and Bone Marrow Cells

Haemocytoblast

Cell size 11–18 μm Non granular cytoplasm, sparse, strongly basophilic Round or oval nucleus Nuclear chromatin evenly distributed, staining reddish purple Two to four distinct nucleoli No attempt was made to further differentiate these cells into myeloid, monocytic, plasma cell or erythroid lines—see Fig 1 a &b

The Myeloid Series

Promyelocyte Cell size 13 18 μm More abundant cytoplasm, constituting about one third of the cell area Bluish colour Distinct reddish purple granules in the perinuclear region Round or oval nucleus, occasionally with a slight indentation Rather coarser chromatin than in the haemocytoblasts Nucleoli still recognizable (Fig 2)

Myelocyte Cell size 12–18 μm. Cytoplasm constituting an increasing proportion of the cell area Distinct neutrophil granules in increasing number in medium—light blue cytoplasm

Beginning differentiation of the nucleus along the two lines which can be followed to the mature cells The *segmented* and the *annular form* The segmented nucleus is slightly indented, the annular form exhibits a central or slightly eccentric hole The chromatin is coarse dark reddish purple No nucleoli are seen (Fig 3)

Metamyelocyte Cell size 8 16 μm The variation in size and other morphology is considerable The typical mid range metamyelocyte has a light bluish, granulated cytoplasm The nucleus of the segmented line cells is clearly indented, kidney shaped while that of the annular line cells has an indented periphery, which gives the nucleus a flower like appearance The chromatin is coarse, occasionally clumped

The metamyelocyte is distinguished from the myelocyte by the degree of nuclear differentiation

Distinction from the mature neutrophil leucocyte was made on the basis of cytoplasmic staining, in that cells showing any degree of basophilia were classified as metamyelocytes (Figs 4–5)

Mature neutrophil granulocyte Cell size 7–10 μm Pink cytoplasm with numerous discrete granules Nucleus segmented or annular, usually with protuberations Chromatin structure dense and coarse (*Fig 6*)

The eosinophil series Apart from eosinophil staining of the cytoplasmatic granules which are also larger and more numerous in this line differentiation was made as described for neutrophils (Fig 7)

The basophil series Cells of this line are not seen in mouse bone marrow nor in the peripheral blood

Lymphopoiesis

Lymphoblasts were included in the haemocytoblast group

Lymphocytes Cell size 7–10 μm The vast majority ('small lymphocytes"), were rounded cells with very sparse light blue cytoplasm, usually occupying only part of the periphery Granules were not distinguishable Round or slightly indented nuclei *Chromatin structure generally fine*, dense Few cells ('large lymphocytes') had more abundant cytoplasm, staining lighter blue with distinct azurophil granules These latter cells were seen in about one per cent of both *nude* and normal mice, and are not listed separately (Figs 8 *9)*

Monocytic Series

Early cells of this series are not distinguished from other early stage cells, but are included in the haemocytoblast group

Mature monocyte Cell size 15–18 μm Cytoplasm greyish blue, sometimes vacuolated and with fine azurophil granules Nucleus bi or multilobulated (Fig 10)

Plasma Cell Series

Early cells again included in the haemocytoblast group

Mature plasma cell Cell size 9–14 μm *Deep* blue, non granular cytoplasm Nucleus small eccentric Dense chromatin (Fig 11)

Megakaryocyte Series

Megakaryocyte Cell size 40–75 μm Abundant blue to light reddish cytoplasm containing numerous *reddish* granules. Nucleus large multilobulated (Fig *12)*

Thrombocyte Size 1–4 μm Light blue cytoplasm, with a central area of azurophil staining material (Fig *13)*

Erythropoiesis

Basophil erythroblast Cell size 8 18 μm Cytoplasm deep blue, non granulated occupying about a quarter of the cell area Usually, cytoplasmic buds were seen at the periphery Nucleus round with unevenly staining chromatin of very dark and coarse pattern *No nucleoli (Fig 14)*

Intermediate and late normoblast Cell size 5–10 μm. A continuous spectrum of maturation Colour of cytoplasm varying from bluish grey to *acidophil*. Nucleus size gradually decreasing with maturation Chromatin more and more *dense Before* extrusion the nucleus may stain quite black (Fig 15

Erythrocyte Cell size 6 7 μm Biconcave and clear Cytoplasm normally orthochrome light red but many polychromatic cells are seen Fig 16)

Histology

Sections were studied by both authors



Fig 17 *Nude* mice of BALB/c (right), C3H (left) and C57/BL background (centre)

Statistical Analyses

Basic statistics and analyses of variance were processed through a Burroughs B 6700 Computer®

RESULTS

Studies of Outward Appearances

Muscle bulk and motor function in the *nude* mice was apparently normal Irrespective of genetic background, all *nudes* look much the same (Fig 17) Most characteristic is the near complete lack of body hair Belts of sparse hair growth may occasionally be seen at various sites over the trunk, a phenomenon most marked in mice of C57/BL background least in those of C3H The whiskers of *nudes* are usually deformed atrophic as described by *Flanagan* (1966)

The skin may be fairly thick and wrinkled or very thin and smooth This study has not taken account of the incidence of these types, and no attempt has been made to identify a possibly responsible genetic factor

Skin pigmentation, however, varies distinctly with genetic background The skin of BALB/c *nudes* is light reddish in colour, and the eyes are albino C3H *nudes* have uniformly light greyish skin, and the eyes are dark pigmented

Skin of C57/BL *nudes* varies from dark greyish to black There is great variation within and between individuals An observed zonal variation is related to the hair growth previously mentioned Pigmentation is most marked in these areas

Autopsy

Macroscopic findings In the following the macroscopic examination of the viscera is described Positive findings are recorded first

Thymus A thymus of normal appearance was found in all normal mice In 22 of the 24 *nudes* sparse light greyish fatty and connective tissue was seen in the superior mediastinum In the remaining two a homogeneous light brown structure, some 2 × 3 × 5 mm, soft and poorly defined, was found at the base of the heart This tissue was taken for histological study

Uterine horns In five of the *nude* mice, the uterine horns were dilated bilaterally—greatest diameter 5 mm—and contained a clear slimy fluid In all other mice, *nudes* and normals, the uterine horns were macroscopically normal

Peyer's patches Varying numbers of Peyer s patches were found in all mice The size of the patches was consistently somewhat smaller in the *nudes*

Other thoracic and abdominal organs There were no apparent differences between the organs of *nudes* and normal mice Posi

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Myelocyte Cell size 12–18 μm. Cytoplasm constituting an increasing proportion of the cell area *Distinct neutrophil granules in increasing number* in medium—light blue cytoplasm

Beginning differentiation of the nucleus along the two lines which can be followed to the mature cells The *segmented* and the *annular* form The segmented nucleus is slightly indented, the annular form exhibits a central or slightly eccentric hole The chromatin is coarse dark reddish purple No nucleoli are seen (Fig 3)

Metamyelocyte Cell size 8–16 μm The variation in size and other morphology is considerable The *typical mid range metamyelocyte has a light bluish*, granulated cytoplasm The nucleus of the segmented line cells is clearly indented, kidney shaped, while that of the annular line cells has an indented periphery, which gives the nucleus a flower like appearance The chromatin is coarse occasionally clumped

The metamyelocyte is distinguished from the myelocyte by the degree of nuclear differentiation

Distinction from the mature neutrophil leucocyte was made on the basis of cytoplasmic staining in that cells showing any degree of basophilia were classified as metamyelocytes (Figs 4 5)

Mature neutrophil granulocyte Cell size 7–10 μm Pink cytoplasm with numerous discrete granules *Nucleus segmented or annular, usually with* protuberations Chromatin structure dense and coarse (Fig 6)

The eosinophil series Apart from eosinophil staining of the cytoplasmatic granules which are also larger and more numerous in this line, differentiation was made as described for neutrophils (Fig 7)

The basophil series Cells of this line are not seen in mouse bone marrow nor in the peripheral blood

Lymphopoiesis

Lymphoblasts were included in the *haemocytoblast* group

Lymphocytes Cell size 7–10 μm The vast majority ('small lymphocytes), were rounded cells with very sparse light blue cytoplasm, usually occupying only part of the periphery Granules were not distinguishable Round or slightly indented nuclei Chromatin structure generally fine dense Few cells ('large lymphocytes) had more abundant cytoplasm, staining lighter blue with distinct azurophil granules. These latter cells were *seen in about one per cent of both nude* and normal mice and are not listed separately (Figs. 8 9)

Monocytic Series

Early cells of this series are not distinguished from other early stage cells but are included in the haemocytoblast group

Mature monocyte Cell size 15–18 μm Cytoplasm greyish blue, sometimes vacuolated and with fine azurophil granules Nucleus bi or multilobulated (Fig 10)

Plasma Cell Series

Early cells again included in the haemocytoblast group

Mature plasma cell Cell size 9–14 μm Deep blue, non granular cytoplasm Nucleus small eccentric Dense chromatin (Fig 11)

Megakaryocyte Series

Megakaryocyte Cell size 40–75 μm Abundant blue to light reddish cytoplasm containing numerous reddish granules Nucleus large multilobulated (Fig 12)

Thrombocyte Size 1–4 μm Light blue cytoplasm, with a central area of azurophil staining material (Fig 13)

Erythropoiesis

Basophil erythroblast Cell size 8 18 μm Cytoplasm deep blue, non granulated, occupying about *a quarter* of the cell area Usually, cytoplasmic buds were seen at the periphery Nucleus round with unevenly staining chromatin of very dark and coarse pattern No nucleoli (Fig 14)

Intermediate and late normoblast Cell size 5 10 μm. A continuous spectrum of maturation *Colour of cytoplasm* varying from bluish grey to acidophil. Nucleus size gradually decreasing with maturation Chromatin more and more dense Before extrusion *the nucleus may stain* quite black (Fig 15)

Erythrocyte Cell size 6 7 μm Biconcave and clear Cytoplasm normally orthochrome, light red *but many* polychromatic cells are seen (Fig 16)

Histology

Sections were studied by both authors



Fig 18 Part of enlarged cystic lymph node from a *nude* mouse Narrow rim of lymphoid tissue sur rounding cystic centre 10 ×, H&E

tion, size, form surface cross section, colour, and consistency were the same

Lymph nodes In the *nudes* much enlarged isolated nodes up to 4 × 6 × 8 mm in size were found among others of normal appearance On section, these large nodes were found to be thin walled, cystic, containing serous yellowish fluid No such node changes were found in the normal mice (Fig 18)

Body weights and organ weights—absolute and relative The average weight for each group of mice is given in Table 1 Tables 1 & 2 give absolute organ weights, and the same weights expressed as a percentage of body weights The numbers of Peyer s patches are appended The weight of the spleen both in absolute and in relative terms was signifi cantly greater, confidence level 99 per cent, in *nudes* of C3H background than in the corresponding normal mice No difference could be demonstrated between spleen weights of BALB/c and C57/BL *nudes*, and normal mice

The Tables 1 & 2 record that lymph node

TABLE 2 *Organ Weights as Percentage of Body Weights*

Strain		Sex	Spleen	Lymph nodes	Thymus	Heart	Lungs	Liver	Kidneys
BALB/c	Normal	F	0 43 (0 06)	0 08 (0 04)	0 28 (0 05)	0 50 (0 06)	0 73 (0 07)	5 18 (0 30)	1 27 (0 03)
		M	0 41 (0 09)	0 06 (0 04)	0 23 (0 13)	0 55 (0 08)	0 65 (0 15)	5 99 (0 38)	1 64 (0 07)
	Nude	F	0 59 (0 16)	0 13 (0 06)	–	0 58 (0 12)	0 75 (0 11)	6 30 (0 40)	1 41 (0 07)
		M	0 55 (0 12)	0 10 (0 02)	–	0 53 (0 01)	0 62 (0 01)	6 24 (0 19)	1 58 (0 11)
C3H	Normal	F	0 30 (0 03)	0 11 (0 06)	0 32 (0 05)	0 47 (0 06)	0 61 (0 05)	5 44 (0 17)	1 17 (0 06)
		M	0 26 (0 03)	0 05 (0 02)	0 25 (0 06)	0 44 (0 01)	0 61 (0 05)	6 20 (0 73)	1 47 (0 10)
	Nude	F	0 54 (0 13)	0 23 (0 07)	–	0 55 (0 03)	0 69 (0 03)	5 80 (0 47)	1 51 (0 11)
		M	0 42 (0 10)	0 15 (0 04)		0 52 (0 05)	0 72 (0 03)	7 01 (0 41)	1 86 (0 15)
C57/BL	Normal	F	0 41 (0 03)	0 11 (0 04)	0 36 (0 05)	0 45 (0 03)	0 76 (0 06)	4 69 (0 36)	1 06 (0 04)
		M	0 35 (0 02)	0 10 (0 06)	0 23 (0 03)	0 47 (0 03)	0 72 (0 02)	5 74 (0 23)	1 13 (0 07)
	Nude	F	0 39 (0 06)	0 23 (0 05)		0 54 (0 09)	0 70 (0 06)	5 59 (0 39)	1 42 (0 08)
		M	0 36 (0 08)	0 21 (0 06)	–	0 47 (0 03)	0 63 (0 04)	5 33 (0 32)	1 36 (0 13)

Mean values of groups of four S E in brackets

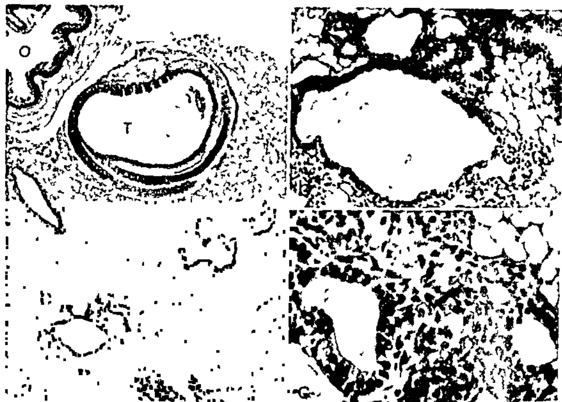


Fig 19 a, b, c 19 a Epithelial partly cystic structures (b, c) in tissue from upper mediastinum of *nude* mouse Structures situated in front of oesophagus (O) and trachea (T), 25 ×, H&E 19 b c Details of epithelial structures seen in Fig 19 a 200 ×, H&E

weight was always greater in the *nudes* than in the normal mice, but the described cystic changes entail that these figures give no reliable expression of the actual quantity of lymphoid tissue present

Other organ weights There was some variation in the absolute weights but no certain differences when these weights were expressed as percentages of body weights, either between *nudes* and normals or between the sexes (Table 2)

Histological Studies
Lymphoid Tissue

Thymus In all the normal mice studied, the thymus was of fine nodular structure with normal proportions of cortex and medulla

In two of the *nudes* some more or less discrete structure was found at the site of a normal thymus The histological study showed irregular tissue predominantly comprising normal fat and brown fat Normal trachea and oesophagus was also seen In one of the preparations, in front of the trachea and oesophagus, one very small island and two larger, of organized tissue were seen The structures were solid, composed of uniform round cells, 10–12 μm in diameter with about 8 μm nuclei The nuclei were uniform, centrally placed and moderately rich in chromatin There were tubular formations in the larger islands, about 50–60 μm in diameter The luminal cells comprised one or two layers of cubical or low columnar epithelium with uniform nuclei In part this epithelium was low ciliated Within the lumina there were sparse aggregations of an homogeneous eosinophil substance (Fig 19) No cells resembling the Hassall cells of the mouse were seen *in* these islands, and not at any site was there seen tissue which in any way resembled thymus tissue

Fig 20 a, b Lymph node of normal mouse, showing germinal centres and a normal amount of lymphocytes in paracortical area (a 25 ×, b 100 ×, H&E)

Fig 21 a, b Lymph node of *nude* mouse, showing broken rim of primary follicles in outer cortex and lymphocytic depletion of paracortical area No germinal centres (a 25 ×, b 100 ×, H&E)

TABLE 3 *Laboratory Values—Peripheral Blood*

			HB (g/100 ml)	RBC $(10^6/\mu l)$	Haematocrit	MCV	MCHC
BALB/c	Normal	F	15 7 (0 3)	11 3 (1 1)	47 (1)	42 (3)	33 (0 5)
		M	15 6 (0 4)	9 3 (1 3)	47 (2)	51 (4)	33 (2)
	Nude	F	13 4 (1 0)	8 8 (0 7)	43 (5)	49 (4)	31 (1)
		M	14 2 (0 3)	9 9 (0 9)	45 (1)	46 (4)	32 (1)
C3H	Normal	F	15 4 (0 4)	10 3 (1 2)	48 (1)	48 (5)	32 (1)
		M	15 0 (0 5)	8 6 (0 7)	48 (1)	55 (4)	32 (2)
	Nude	F	13 6 (0 6)	8 0 (0 9)	42 (3)	53 (4)	33 (1)
		M	12 8 (0 3)	6 9 (0 9)	40 (2)	58 (4)	33 (1)
C57/Bl	Normal	F	14 4 (0 2)	9 4 (0 4)	47 (2)	50 (1)	31 (1)
		M	14 7 (0 9)	9 8 (0 5)	49 (2)	50 (3)	31 (1)
	Nude	F	14 4 (1 0)	8 7 (0 4)	46 (3)	52 (5)	31 (1)
		M	13 5 (0 1)	8 6 (0 8)	43 (1)	51 (5)	32 (1)

Mean values of groups of four S E in brackets

Lymph nodes (The terminology adopted in this study is as proposed by *Cottier et al* 1972)

The nodes studied from normal mice of the three strains were similar, and of normal structure (Fig 20)

Nodes from *nudes* of the three genetic backgrounds were also similar in appearance, but varied to greater or lesser degree from the normal structure (Fig 21)

Size The microscopic study confirmed the macroscopic impression that nodes from *nudes* were consistently larger than nodes from the normal mice The largest nodes were the most cystic, but gross structure was otherwise preserved (Fig 18)

Node capsules were of normal appearance

Cortex (outer cortex) Immediately under the subcapsular sinus the cortex appeared as a small frequently broken rim formed of uniform small lymphocytic cells There were occasional larger lymphoid cells and plasma cells The postcapillary venules were present in normal numbers In the same region there were lymph follicles comprising dense collections of small lymphocytes but no germinal centres

Paracortical area (inner cortex) This area lay adjacent to the capsule and outer cortex peripherally and extended centrally to the medullary cords There were very few small lymphocytes Tissue structure was loose comprised of reticulum cells and endothelial cells of the lymphatic sinuses Some histiocytes were seen The postcapillary venules were normal Lymphocyte diapedesis was observed in *nude* nodes in both the inner and outer cortex, but to somewhat lesser extent than in normal lymph nodes

Medullary cords The cords were generally poorly differentiated from the paracortical



Fig 22 a b a. Peyer's patch from normal mouse showing lymphoid tissue with germinal centre (GC) between tunica serosa (S) and tunica mucosa (M) 100 × H&E b Detail of germinal centre of the same section Abundant phagocytosis in macrophages (arrows) 400 × H&E

Fig 23 a b a Peyer's patch from *nude* mouse Marked lymphocytic depletion and no germinal centres 100 ×, H&E b Detail of the same section Macrophages present (arrows), but without phagocytic activity 400 ×, H&E

TABLE 3 *Laboratory Values—Peripheral Blood*

			HB (g/100 ml)	RBC ($10^6/\mu l$)	Haematocrit	MCV	MCHC
BALB/c	Normal	F	15.7 (0.3)	11.3 (1.1)	47 (1)	42 (3)	33 (0.5)
		M	15.6 (0.4)	9.3 (1.3)	47 (2)	51 (4)	33 (2)
	Nude	F	13.4 (1.0)	8.8 (0.7)	43 (5)	49 (4)	31 (1)
		M	14.2 (0.3)	9.9 (0.9)	45 (1)	46 (4)	32 (1)
C3H	Normal	F	15.4 (0.4)	10.3 (1.2)	48 (1)	48 (5)	32 (1)
		M	15.0 (0.5)	8.6 (0.7)	48 (1)	55 (4)	32 (2)
	Nude	F	13.6 (0.6)	8.0 (0.9)	42 (3)	53 (4)	33 (1)
		M	12.8 (0.3)	6.9 (0.9)	40 (2)	59 (4)	33 (1)
C57/Bl	Normal	F	14.4 (0.2)	9.4 (0.4)	47 (2)	50 (1)	31 (1)
		M	14.7 (0.9)	9.8 (0.5)	49 (2)	50 (3)	31 (1)
	Nude	F	14.4 (1.0)	8.7 (0.4)	46 (3)	52 (5)	31 (1)
		M	13.5 (0.1)	8.6 (0.8)	43 (1)	51 (5)	32 (1)

Mean values of groups of four. S.E. in brackets.

Lymph nodes (The terminology adopted in this study is as proposed by *Cottier et al.* 1972.)

The nodes studied from normal mice of the three strains were similar and of normal structure (Fig. 20).

Nodes from *nudes* of the three genetic backgrounds were also similar in appearance, but varied to greater or lesser degree from the normal structure (Fig. 21).

Size. The microscopic study confirmed the macroscopic impression that nodes from *nudes* were consistently larger than nodes from the normal mice. The largest nodes were the most cystic, but gross structure was otherwise preserved (Fig. 18).

Node capsules were of normal appearance.

Cortex (outer cortex). Immediately under the subcapsular sinus the cortex appeared as a small, frequently broken rim formed of uniform small lymphocytic cells. There were occasional larger lymphoid cells and plasma cells. The postcapillary venules were present in normal numbers. In the same region, there were lymph follicles comprising dense collections of small lymphocytes, but no germinal centres.

Paracortical area (inner cortex). This area lay adjacent to the capsule and outer cortex peripherally, and extended centrally to the medullary cords. There were very few small lymphocytes. Tissue structure was loose, comprised of reticulum cells and endothelial cells of the lymphatic sinuses. Some histiocytes were seen. The postcapillary venules were normal. Lymphocyte diapedesis was observed in *nude* nodes in both the inner and outer cortex, but to somewhat lesser extent than in normal lymph nodes.

Medullary cords. The cords were generally poorly differentiated from the paracortical



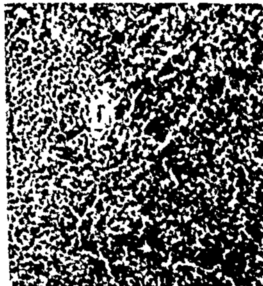
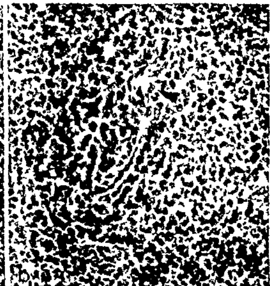



Fig 24 a, b Spleen from normal mouse Malpighian body with large number of lymphocytes in peri arteriolar area Central arteriole at arrow (a 100 ×, b 250 × H&E)

Fig 25 a, b Spleen from *nude* mouse Malpighian body with marked lymphocytic depletion in peri arteriolar area Central arteriole at arrow (a 100 ×, b 250 ×, H&E)

region Rarely, well marked cords were seen, predominantly associated with immature plasma cells The reticulum cells were interspersed with large lymphoid cell types in addition to the plasma cells

Lymphatic sinuses (subcapsular sinus intermediate sinus and medullary sinus) These structures were of normal appearance

Peyer's patches (small intestine) Peyer's patches in the normal mice were of normal size and appearance (Fig 22)

The appearance in the *nude* mice was uniform, irrespective of genetic background but different from that in the normal mice

The patches were consistently smaller, but location and distribution between the lamina propria the tunica mucosa and the tunica adventitia was normal

The structural tissue was loose predominantly composed of reticulum cells and endothelial cells which defined the sinusoids Blood vessels were of normal appearance Isolated small lymphocytes were seen randomly in this tissue, while centrally there were denser round accumulations of small lymphocytes which sometimes included structures resembling secondary follicles (germinal centres), though much smaller than the follicles seen in normal mice A constant characteristic of these follicles in the *nudes* was the absence of or marked reduction in, phagocytic activity It was always easy to differentiate between Peyer's patches from normal mice and *nude* mice on this ground alone (Fig 23)

Peyer's patches from the colon when present, were as described in the small gut

Lamina propria The diffuse lymphoid tissue in the lamina propria of the small bowel was similar in constitution and quantity in both *nudes* and normal mice

Spleen The spleen was normal in the normal animals

In the *nudes* the appearance was uniform but differed from that in the normal animals in some respects

Distribution of red and white pulp was similar to that in the normal mice and no certain qualitative changes could be seen not even in the degree of extramedullary haemopoiesis in the red pulp The Malpighian bodies however, differed in two respects

a) The marginal zone of medium size lymphocytes comprised 10–15 cell layers in *nudes* only 2–6 layers in the normal mice (Figs 24–25)

b) The periarteriolar reticulum cell structures were virtually devoid of lymphoid cells (Figs 24–25)

Bone Marrow

The histological studies of the marrow containing bones indicated that the extent of haemopoiesis was much the same in *nudes* and normal mice The marrow in the cranium sternum ribs spine and the long bones of the extremities was maximally active Haemopoiesis in the tail bones varied somewhat, but was generally sparse Cellular constitution appeared much the same and no unusual elements were observed

Differential counts from bone marrow (Table 6) No morphological differences in the cell lines were observed Particularly the plasma cells were of similar appearance The differential counts (Table 6) demonstrated that numbers of lymphocytes were consistently lower in the *nudes* The differences are significant with a confidence level of 95 per cent (Student's t test) No quantitative differences in myelo- or erythropoiesis were evident

Peripheral Blood (Table 5)

All the *nude* mice evidenced leucopenia—between 40 and 90 per cent of the counts in the normal mice The range of means in the normal mice was 2200–6690 leucocytes/μl and in the *nudes*, 690–6990 leucocytes/μl No significant sex related differences were observed

May Grunwald—Giemsa stained preparations showed no qualitative differences between the peripheral blood cells of *nude* and normal mice The differential counting of 200 nucleated cells after the previously recorded criteria gave the distributions recorded in Table 5 The *lymphocyte counts*

TABLE 5 *White Blood Cell Counts in Peripheral Blood Total and Differential Counts*

Strain		Sex	Count/µl WBC	Min	Max	Gran*	Min	Max	Lymph*	Min	Max	Per cent Gran*	Per cent Lymph*
BALB/c	Normal	F	4302 (1539)	2200	5880	1290 (729)	396	2000	3012 (885)	1804	3880	28.0	72.0
		M	3635 (808)	2720	4680	854 (240)	570	1125	2781 (579)	2150	3555	23.3	76.7
	Nude	F	2380 (641)	1760	3260	1134 (688)	530	2120	1216 (303)	880	1580	45.5	54.5
		M	3138 (485)	2700	3620	1675 (287)	1380	1955	1463 (217)	1260	1735	53.3	46.7
C3H	Normal	F	3695 (1053)	2730	5060	762 (340)	516	1265	2933 (818)	2100	3795	20.5	79.5
		M	3983 (879)	2740	4800	1654 (565)	1280	2495	2329 (718)	1340	3000	42.0	58.0
	Nude	F	2248 (629)	1400	2750	954 (300)	546	1220	1294 (475)	854	1780	42.8	57.2
		M	2360 (623)	1820	2990	1078 (268)	680	1260	1282 (486)	655	1730	46.6	53.4
C57/BL	Normal	F	5678 (1083)	4160	6690	713 (259)	345	940	4965 (1103)	3330	5750	13.0	87.0
		M	4783 (536)	4390	5550	601 (133)	475	780	4182 (443)	3820	4770	12.5	87.5
	Nude	F	2245 (1044)	690	2880	889 (412)	352	1350	1356 (751)	338	1945	42.0	58.0
		M	3780 (1486)	2830	5990	1373 (500)	965	2100	2407 (993)	1830	3890	36.5	63.5

Mean values of groups of four S.E. in brackets
* Abbreviations Gran = granulocytes Lymph = lymphocytes and monocytes

TABLE 6 *Differential Counts of Bone Marrow Cells**

Strain		Sex	Blast	Promy	Myneu	Myeo	Meta	Graneu	Graneo	Lymph	Pl C	Mono	Mega	Baser	IMR
BALB/c	Normal	F	3 0	1 0	2 5	0 6	17 4	20 9	2 5	16 3	0	0 1	1 0	3 6	31 1
			(1 5)	(0 7)	(1 0)	(0 8)	(4 6)	(3 4)	(1 2)	(1 1)		(0 3)	(0 8)	(2 1)	(8 9)
		M	5 6	0 1	3 1	0 1	12 8	29 5	1 6	11 0	0 3	0 3	0 3	2 8	32 5
			(3 5)	(0 3)	(1 0)	(0 3)	(1 4)	(1 2)	(1 1)	(4 8)	(0 5)	(0 3)	(0 3)	(1 2)	(6 6)
	Nude	F	2 3	0	1 9	0	17 5	23 5	5 5	6 6	0	0	0 3	1 8	40 6
			(2 2)		(1 7)		(6 1)	(4 9)	(3 2)	(4 2)			(0 3)	(1 9)	(4 3)
		M	3 0	0 1	2 4	0	16 5	33 2	4 9	1 5	0	0	0 1	3 0	35 3
			(1 2)	(0 3)	(1 7)		(7 5)	(7 6)	(3 5)	(1 0)			(0 3)	(0 8)	(3 1)
C3H	Normal	F	6 5	1 4	1 3	0 3	12 1	23 1	1 6	11 5	0 1	0	0 4	6 4	35 3
			(2 2)	(0 9)	(0 9)	(0 5)	(2 2)	(10 4)	(1 4)	(3 5)	(0 3)		(0 3)	(2 9)	(7 0)
		M	5 6	0 3	5 6	0	14 6	20 9	1 9	10 9	0	0	0 1	3 1	37 0
			(2 0)	(0 3)	(3 0)		(4 7)	(0 8)	(0 9)	(2 6)			(0 3)	(1 1)	(6 1)
	Nude	F	6 3	0 5	3 3	0 1	17 0	22 5	1 4	7 0	0 1	0	0	3 0	38 8
			(2 8)	(0 7)	(2 4)	(0 3)	(6 0)	(6 7)	(0 8)	(4 3)	(0 3)			(2 1)	(9 7)
			7 0	0 5	1 6	0 3	16 1	26 1	2 4	9 1	0 4	0	0 4	1 4	31 7
			(2 9)	(0 4)	(1 1)	(0 3)	(4 3)	(4 2)	(1 6)	(2 4)	(0 5)		(0 5)	(1 8)	(9 3)
C57/BL	Normal	F	4 8	1 3	2 1	0 3	17 3	16 6	1 1	16 8	0	0	0 1	2 0	34 3
			(0 6)	(1 0)	(1 3)	(0 5)	(4 4)	(0 8)	(2 8)	(3 1)			(0 3)	(0 8)	(5 0)
		M	5 4	0 3	3 8	0 1	13 9	21 2	3 1	9 9	0	0	0 1	2 9	39 3
			(0 6)	(0 3)	(1 7)	(0 3)	(2 0)	(3 9)	(2 1)	(5 2)			(0 3)	(1 7)	(4 1)
	Nude	F	4 5	0 6	4 1	0 1	17 5	31 0	1 4	7 9	0	0	0 3	1 9	30 7
			(1 9)	(0 5)	(3 0)	(0 3)	(1 9)	(5 9)	(0 9)	(1 1)			(0 3)	(0 3)	(11 8)
		M	5 5	0 8	3 1	0 3	19 8	20 4	2 3	10 1	0	0	0 1	0 9	36 7
			(4 7)	(0 3)	(2 6)	(0 3)	(5 4)	(6 0)	(1 0)	(5 2)			(0 3)	(0 5)	(1 6)

Mean values of groups of four S E in brackets

* Abbreviations *Blast* = *haemocytoblast*, Promy – promyelocyte Myneu – neutrophil myelocyte Myeo = eosinophil m Meta = metamyelocyte, Graneu = neutrophil granulocyte Graneo – eosinophil g Lymph – lymphocyte Pl C = plasma cell, Mono = monocyte, Mega = megakaryocyte Baser – basoph l *erythroblast IMR intermed ate and late normoblast*

TABLE 7 *Analyses of Variance of Lymphocyte and Granulocyte Counts in Peripheral Blood*

	Lymphocytes		D freedom	Granulocytes	
	F value	Confidence level %		F value	Confidence level %
BALB/c *nude* vs normal	33 53	100 00	1,14	1 41	74 46
C3H *nude* vs normal	17 81	99 91	1,14	0 60	not computed
C57/BL *nude* vs normal	32 90	99 99	1 14	6 27	97 48
All *nude* vs all normal mice	46 07	100 00	1,46	1 97	83 26
Nude mice of one genetic background vs all *nude* mice	2 03	84 36	2 21	1 49	75 17
Normal mice of one stra n vs all normal mice	14 09	99 99	2 21	2 60	90 18

Variance between *nude* and normal mice within strains and total between *nude* mice of different genetic backgrounds and between normal mice of corresponding inbred strains

differed markedly from group to group The percentage proportions are given in the table The appended absolute counts are, however, of greater interest The consistent average *count in nudes was about 1400/µl, only 28 to* 58 per cent of the numbers of lymphocytes in the normal mice The range of means in the normal mice was 1340 to 5750 lymphocytes/µl, and in the *nudes,* 338 to 3890 lymphocytes /µl

Analyses of variance were performed to test

a) The variation of lymphocyte counts between *nudes* and normals within strain (i e inbred mice and mice of corresponding genetic background)

b) The variation of lymphocyte counts between *nudes of different genetic backgrounds*

The results are given in Table 7, and indicate

a) That our *nudes* and normal mice of the same genetic background do *not* belong to the same population, as judged by lymphocyte counts, and

b) that all *nudes* could well belong to the same population

Similar analyses of variance were performed with the *granulocyte counts*

Results are given in Table 7

These indicate that all the granulocyte counts could stem from the same population

Histological Studies of Organs and Tissues

Normal mice No pathological changes were demonstrated in any organ or tissue

Nude mice In 11 of the *nudes,* non specific inflammatory changes, sometimes with abscess formation, were found to involve one or more organs

Harderian glands	7 mice
Uterine horns	5 mice
Seminal vesicles	1 mouse
Parotid glands	1 mouse

The histological appearance of the inflammation of the uterine horns is recorded in Fig 26 These pathological changes involved *nudes* of all genetic backgrounds

No other pathological changes were seen in any of the *nudes*

Laboratory Studies—Blood

Haemoglobin Concentration, Erythrocyte Counts, Haematocrit, MCV and MCHC

Results are given in Table 3

Haemoglobin *concentration,* erythrocyte counts and heamatocrit were somewhat lower in the *nudes* (not significantly) There was no certain variation with genetic background

Serum Proteins, Total and Fractionated

As shown in Table 4, there were no significant differences between normal mice and

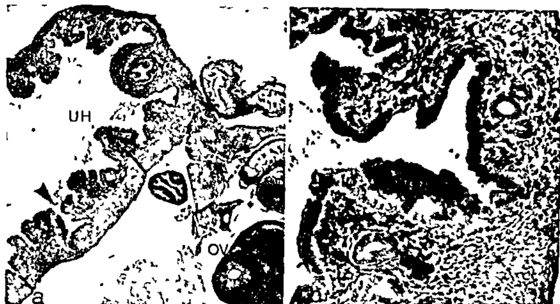


Fig 26 a, b a Normal ovary (OV) and strongly dilated uterine horn (UH) of *nude* mouse 15 ×, H&E b Detail of a (at arrow) Wall of uterine horn with heavy non specific inflammatory changes 100 ×, H&E

nudes in total serum protein nor in albumin, alpha 1, alpha 2, beta and gamma fractions

DISCUSSION

Physical Appearances

All *nudes* homozygous for the *nu* gene, irrespective of their genetic background, BALB/c, C3H, or C57/BL were absolutely distinctive in their lack of hair, or their sparse zonal hair growth

Skin pigmentation was similar to that in the inbred strains to which the gene was under transfer The degree of similarity, which was greater than expected at the third cross stage reflects a selection of breeding animals after colour in each of the two breed ing cycles before the present study

Body Weights—Organ Weights

Table 1 records that female weight was consistently less than that of male mice

Nude weights were sometimes higher than those of the corresponding normal mice, which finding conflicts with others' reports (*Flanagan* 1966) and with our general ob servations (*Rygaard & Friis* 1973) The probable explanation is that the numbers are small and the age range (6–8 weeks) is at a critical level Within this age range there is considerable variation in weight, and in weight gain (*Rygaard & Friis* 1973)

Organ weights, absolute and related to body weight were much the same in both *nudes* and normal mice with two exceptions both concerned with immune responses

a) The *spleens* in *nudes* of BALB/c and C3H background, and

b) *lymph nodes* in *nudes* of all three back-grounds, were of *greater* weight than in the normal mice

This is an opposite finding to that of *De Sousa et al* (1969) They report markedly smaller spleens in homozygous *nu/nu* mice than in their phenotypically normal litter mates (+/*nu* or +/+) and *lymph nodes* of uniform size, or slightly smaller in the *nudes* This may be explicable in terms of different genetic backgrounds and/or breeding milieu The frequently cystic nodes in the *nudes* of our study explain the higher weight, but are by no means an indication of the actual quantity of lymphoid tissue in the lymph nodes of the *nude*

Autopsy Findings

All animals were of healthy external appearance. Internally, no pathological changes were found in normal mice, but 11 of the 24 *nudes* (46 per cent) had changes in one or more organs.

The infections were always canalicular, in the Harderian glands, the parotids, and the genitalia.

The genital changes occurred predominantly in females, in 5 of 12 animals (42 per cent). One of 12 males had seminal vesicle infection.

The uterine horn infections partly explain the poor fertility of female *nudes*. The lesser incidence of infection in males may explain the excellent fertility of male *nudes* (90–95 per cent, *Rygaard* & *Friis* 1973).

The differential bone marrow and peripheral blood counts in the infected *nudes* were not different from these in the healthy *nudes*.

All animals were reared under so called spf (specified pathogen-free) conditions. No normal mice evidenced any infection.

The infections in the *nudes* were probably caused by normally not pathogenic organisms, and reflect the impaired resistence capacity of the animals.

Thus it is evident that the normal implications of spf rearing are not valid in *nudes*. *Friis* (personal communication) found the agent of infection in a series of *nudes* to be the normally non pathogenic *Staph. albus*. Despite the finding of infection in some of our *nude* mice, the foregoing considerations plus the fact that the actual rearing milieu must be among the best, seemed to indicate that these mice should be included in the study.

An spf milieu defined with reference to the *nude* would entail a considerable extension of the number of specified pathogens, probably ultimately a list of thymus dependent, normally not pathogenic organisms in addition to the recognized pathogens.

Crewther & *Warner* (1972) report very low serum IgA values in *nudes*—between 5 per cent and 7 per cent of that value in normal mice. *Jutila* & *Reed* (1972) found little or no IgA in *nude* mouse faeces. They take this to explain the gastro intestinal infections frequent in *nudes*, probably caused by usually non-pathogenic coliforms. No studies have been published, as yet, of IgA in tears, saliva, and secretions of the genital tract, but low content of the immunoglobulin would explain the frequent ascending infections to which *nudes* are prone, and secondarily, the frequent infertility in the animals. Such studies are in progress.

Histological Findings

The thymus was normal in all the normal mice.

No thymus tissue could be identified in any of the *nudes*. In two animals apparently discrete tissue in the superior mediastinum was composed of cystic and organized solid aggregations. No lymphoid tissue could be found, and nothing resembling the epithelial structure of the normal thymus was seen. Similar findings were made by *Wortis et al.* (1971), and there is no great variance from the descriptions given by *Pantelouris* & *Hair* (1970).

In summary, there are no morphological grounds for supposing that post natal *nudes* have any functional thymus tissue at all. It is even uncertain whether it is justifiable to describe the mediastinal tissue aggregations as remnants of the thymic rudiments which have been described (*Pantelouris* & *Hair* 1970, *Holub*, personal communication). *Holub* does not think so, and as *Wortis et al.* (1971) opine, the tissue may be a remnant of the ultimobranchial bodies or epithelial bodies. Further elucidation is required.

Lymph Nodes

Nodes were of normal structure in all normal mice, but in all *nudes*, irrespective of genetic background, the paracortical area was depleted, and secondary follicles were not developed. This is as seen in mice after neonatal thymectomy (*Parrott et al.* 1966), and as described by others in other *nudes* (*De Sousa et al.* 1969). The reasons for the cyst formations in our *nudes* are not apparent.

Peyer's Patches

Peyer's patches in the normal mice were without pathological changes In the *nudes* the appearance was roughly as described by *De Sousa et al* (1969), but the lymphocytic depletion seems to have been yet more marked in ours This probably reflects the rearing of our animals under spf conditions De Sousa s were conventionally reared

In addition to the changes which have been described earlier, we observed a clear difference in the degree of the phagocytic activity of macrophages In normal mice, large numbers of phagocytic cells containing phagocytosed material (speckled appearance) were seen This intracellular appearance was virtually not seen in the *nudes* As earlier mentioned, this was a characteristic finding which allowed immediate differentiation between *nudes* and normal mice At this stage, it cannot be determined whether this lack of phagocytic activity reflects differences in the immunoglobulins, specifically in IgA (*Crewther & Warner 1972, Jutila & Reed 1972, Salomon & Bazin 1972*) *Crewther & Warner* found *in vitro* synthesis of IgA from *nude* Peyer's patch fragments

In vivo synthesis and the functional capacity of the IgA, is obscure and awaits further study, but the morphological abnormalities strongly suggest functional abnormality

Spleen

The spleens of the normal mice were normal in appearance and development, but in the *nudes* irrespective of genetic background, there was deficient development of the so called thymus dependent lymphoid tissue in the peri arteriolar regions This has been described previously in neonatally thymectomized mice (*Parrot et al* 1966) and in other *nudes* (*De Sousa et al* 1969)

Additionally we record that the marginal zone of the Malpighian bodies was of markedly greater breadth in all the *nudes*

The histological study gave no explanation of the consistently greater weight of the spleen in the *nudes*

Bone Marrow

Morphologically the marrow of *nudes* and normal mice was similar, but the differential counts exposed a difference in cellular composition Thus lymphoid cell counts in *nudes* were consistently lower than in normal mice Supposing that all these *nude* cells were B lymphocytes, and that T- and B lymphocyte populations are independently regulated, the count difference between *nudes* and normal mice may be an expression of the T cell fraction in the latter If this is so, the T-fraction constitutes between three and 10 per cent of the total lymphoid count, which is as suggested by other studies (*Raff* 1971)

The marrow differential count in normal mice was in full accordance with the findings of some authors (*Endicott & Gump* 1947, *Jaffé* 1958), but at variance with others (*Dumas* 1953, *Langendorff & Popperitz* 1939) In this context, *Jaffé* (1958) for example, points the difficulties inherent in any comparisons, occasioned by differences in nomenclature and definitions of the individual cell types We therefore present this study with definitions, and with microphotographs, which will facilitate any future comparisons The method of preparation—delicate washing and cytocentrifuging—is one we would strongly recommend Cell damage is negligible and appearances are clear—in particular the obscuring serum overlay is avoided Direct spreading of bone marrow in serum on slides is an inferior method in our hands Comparisons were made, and the centrifuge method gave preparations which were a much better basis for unambiguous classification

Peripheral Blood

The white blood cell counts in mice of the inbred strains varied somewhat C3H mice had the lowest counts and C57/BL the highest The differential count was as found by others (*Russel & Bernstein* 1966)

Lymphocyte counts in all *nudes*, irrespective of genetic background, were constantly around 1400/μl This uniformity, considered together with the considerable variations ob

served in the normal mice, suggests a lymphocyte fraction without relation to the thymus, which is regulated by an autonomous homeo static system This fraction must be the B-lymphocytes

If the B lymphocyte population in normal mice corresponds with that in *nudes*, the additionally counted cells in the normal mice must be T-lymphocytes This implies that the T-cell population is variable, unlike the B cell population The studies made by *Raff* (1971) and others with anti theta demonstration of T-cells and anti Ig demonstration of B cells are entirely consistent with this implication

These comparisons between lymphocytes of *nudes* and respective inbred normal mice must be regarded with some reservation, in that the study concerns mice at a stage in gene transfer, not an isogenic population On completion of the gene transfer, a further comparison of lymphocytes should give more positive information about the T-cell fraction variation suggested by this present study

No other marked haematological differences between *nudes* and normal mice became evident It is postulated that the defect in the *nude* might reflect absence of a primitive stem cell precursor of the T-cells Alternatively if this basic cell is present, and is also the precursor of myeloid and erythroid lines, then the T-cell differentiation must be specifically arrested, and the defect not reflected by the other cell lines

Th [illegible]

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the *nude* has normal stem cells which can differentiate to give T cells in epithelial thymic remnants of normal mice which have been exposed to sub lethal irradiation

Serum Proteins

There were no apparent differences between *nudes* and normal mice in total protein or fractionated protein—specifically not in the gamma fraction This is an observation rather similar to that made by *Fahey et al* (1965) and *Humphrey et al* (1964) in neonatally thymectomized mice It is noted that the fractionated protein study method is in sufficiently sensitive to detect, say small differences in the immunoglobulin subfraction of the gamma globulins Such difference has been demonstrated previously by immunochemical methods (*Rygaard* 1969, *Salomon* & *Bain* 1972, *Wortis* 1971, *Luzzati* & *Jacobson* 1972, *Crewther* & *Warner* 1972), which are being applied in a continuation of the basic study herein reported

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We wish to thank *J Weis Fogh* M D, of the Department of Clinical Chemistry, Sundby Hospital, for valuable advice and for access to his laboratory Miss *Inger Bull Birgitte Bigom*, *Margit Bæksted* gave willing and expert assistance, and Mr *Oluf Rasmussen* assisted in a expert way *Michael Gold schmidt*, M Sc, led us gently through our statistical problems The cost of printing the coloured plate was covered by A/S Ulf Olsen, Copenhagen the Danish representative of Carl Zeiss Jena

The study was supported by *The Danish Cancer Society The Danish Medical Research Council* and *P Carl Pedersens Fond*

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OBSERVATIONS REGARDING THE SPECIFIC POST-CAPILLARY VENULES OF LYMPH NODES IN MALIGNANT LYMPHOMAS

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The prevalence of postcapillary venules with high endothelium cells (HE venules) in lymph nodes was studied in biopsy specimens from 18 cases of malignant lymphoma HE venules were found to be absent in non leukaemic lymphocytic and lymphoblastic lymphomas, usually absent but in some cases even abundant in chronic lymphatic leukaemia and finally present and often conspicuous in the specific tissue of Hodkin s disease (lymphocyte predominance mixed cellularity and nodular sclerosis) In a discussion of the observation it is noted that it can still not be taken for granted that HE venules serve the immigration of recirculating lymphocytes to the lymphatic parenchyma and that the biology of these curious vessels remains to be settled

Post capillary venules with high endothelium cells (HE venules) have long been known as a specific structural detail of the diffuse cortex (paracortical zone) of lymph nodes Viable lymphocytes apparently lodge *w thin* the cytoplasm of these endothelium cells, observations by *Gowans & al* (1964) made it a popular concept that they are recirculating blood lymphocytes in passage through the venular lumen for the lymphatic parenchyma HE venules have been devoted little attention in histopathology, probably due to the fact that obturation of the lumen by the swollen endothelial cells and infiltration by lymphocytes tend to obscure the vascular pattern of HE venules in the thick section often used in routine diagnostic work The present study is an attempt to define the prevalence of HE venules in lymph nodes invaded by various types of malignant lymphoma, using *thin sections,* in which HE venules are distinct and conspicuous

Received 12 vii 73 Accepted 12 vii 73

MATERIAL AND METHODS

Biopsy specimens of lymph nodes were obtained from 18 patients with a clinical and histological diagnosis of the following types of malignant lymphoma

1 *Non leukaemic lymphocytic and lymphoblastic lymphoma* (4 cases), corresponding to the classical labels lymphosarcoma and reticulum cell sarcoma There was no case of true histiocytic lymphoma The specimens were all obtained from the material on which the first diagnosis had been established There were three cervical and one inguinal specimens No treatment had been given before the biopsy The age of patients was in all cases above 40

2 *Chronic lymphatic leukaemia* (8 cases) The age of all patients was above 50 and they all presented the classic clinical picture of uncomplicated CLL a white cell count of 80 000 150,000 cells, of which more than 80 per cent were mature lymphocytes more or less enlarged lymph nodes

in several stations and more than 50 per cent lymphocytes in the bone marrow smear The diagnosis had been established two months—three years before the present biopsy which in six cases had been obtained from the cervical, in one case from the inguinal and in one case from the axillary region Three of the patients had previously been given corticosteroids and one had even been given chlorambucil but all had been without specific *treatment for three months before biopsy*

3 *Hodgkin's disease* (6 cases), with subclassification according to *Lukes lymphocytic predominance (2 cases), mixed cellularity (3 cases)* and *nodular sclerosis* (1 case) The specimens studied had been obtained at intervals ranging from about one month up to slightly more than one year after the time of the first diagnosis Five specimens were cervical one from the abdomen (mesenteric?) Three patients had passed one seance of regional radiotherapy, not directed against the region from which the present specimens had been obtained, none had received cytostatic drugs or corticosteroids

Two five nodes were obtained from each case, often in a single tissue block, no node was more than 1 5 cm in diameter Various numbers of thin tissue blocks were prepared from each node, fixed in glutar aldehyde, after treated with osmium tetroxyde and embedded in epon 1 μ sections were studied in phase contrast unstained, stained with May Grunwald Giemsa or silver impregnated according to Movat Material prepared for electron microscopy will not be referred to in this paper

Some general facts given in this paper regarding the HE venules in non neoplastic lymphatic tissue of man are based on an extensive material of thin section specimens collected in this laboratory

OBSERVATIONS

A *Some Background Facts Regarding HE Venules in Man*

HE venules may be defined as small venules with very bulky endothelial cells, sometimes obturating more or less completely the lumen in which small lymphocytes usually occur much in excess of the numbers to be expected from its content of red cells In addition lymphocytes or clusters of lymphocytes are normally seen *within* and between the endothelium cells and between these cells and the basement membrane which is normally distinct and often rather thick, in rare cases, lymphocytes are seen in the act of passing through this membrane On the other hand, a special sheath or cuff of venule oriented lymphocytes is *sometimes seen outside* the basement membrane, poorly demarcated against the solid paracortical parenchyma, but normally always separating the venules from adjacent lymph tracts (*Söderström 1967*)

HE venules form the histological landmark of the paracortical or thymus-dependent zone of lymph nodes In man they are always present in lymph nodes and gut associated lymphatic tissue, their numbers reflect the activity of the tissue and they are thus conspicuously abundant *in tonsils and mesenteric* nodes In the spleen and the normal thymus HE venules are never seen, but they may turn up together with germinal centres in "thymitis", e g in myasthenia (*Söderström & al 1970*) *In ectopic lymphatic tissue (e g* in Hashimoto goiters) we have seen them only in the vicinity of germinal centres

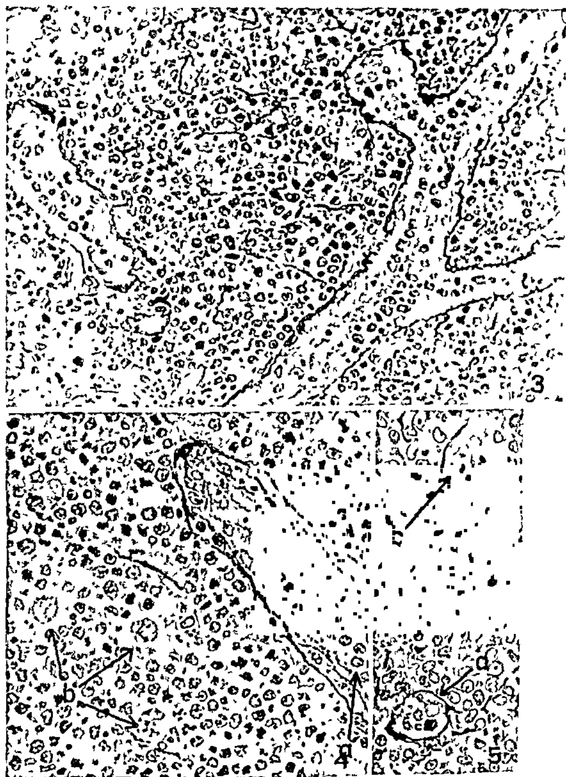
B *HE Venules in Different Types of Malignant Lymphoma*

1 *Non leukaemic lymphocytic and lymphoblastic lymphoma* In all specimens from the four cases in this group, the lymphoma tissue had the organization of a homogenous tumour tissue which in the thin section picture appeared very different from that of the lymph node Irregular thin walled venules were seen but *no HE venules* Only in one *specimen a narrow zone of* residual lymph node tissue was present outside the sharply demarcated lymphoma, it was immediately

Fig 1 Lymph node tissue of the type lymphocytic lymphoma from one of the cases of CLL No HE *venules were found in this type of* tissue the venules with thin endothelium are similar to those seen in the medulla of thymus (1 μ section toluidine blue, × 240)

Fig 2 Lymph node section from one of the cases of CLL Diffuse loss structural differentiation no HE venules are visible Some remnants *of the* original lymph node architecture revealed by the silver impregnation, appear to serve no purpose in this tissue, diffusely flooded by *lymphocytes* (1 μ section silver impregnation according to Movat × 100)

2


3
b
a
4
m
d
5

recognized by the presence of distinct HE venules

2 *Chronic lymphatic leukaemia (CLL)* In this group three different patterns could be distinguished with respect to the prevalence of HE venules

a In two cases, sharply demarcated foci of lymphoma tissue were found which repeated the histological picture of the non leukaemic lymphocytic lymphomas They were rich in thin walled vessels (venules or/and lymph vessels) (Fig 1), but HE venules were seen only outside these distinct nodules and signalled, in a very conspicuous way, areas still not invaded by the lymphoma tissue

b In four cases the specimens presented a classic histological picture of CLL, with a monotonous carpet of small lymphocytes all over the node, infiltrating locally also the extracapsular tissue Secondary nodules and plasma cells were absent and lymph sinuses often crowded with lymphocytes, were seen only in the subcapsular region Thin walled venules were seen, but HE venules were not present or at most represented by single widely scattered specimens In these specimens silver impregnation sometimes revealed vestigial background patterns corresponding to a normal lymph node structure, i a annular formations which might represent basement membranes of venules once present in this tissue now flooded by leukaemic lymphocytes (Fig 2)

c In the two remaining cases, HE venules were more or less abundant in all the specimens studied (Fig 3) In both cases, HE venules could be seen in direct contact with lymph sinuses without interposition of a zone rich in lymphocytes, in other respects the HE venules appeared normal In both cases, a typical clinical CLL had been present for more than one year before the biopsy with moderately enlarged lymph nodes in the cervical and axillary regions, the course of the disease appeared to be rather benign and suggested no specific features All the nodes studied were enlarged, secondary nodules and plasma cells were absent and lymphocytic infiltrates extended outside the capsule Conventional criteria supporting a histological diagnosis of CLL were thus at hand

3 *Hodgkin s disease* In all specimens obtained from the six cases of Hodgkin's disease, HE venules were present and in some of them even in abundance In the cases labelled lymphocyte predominance and mixed cellularity, typical HE venules, rich in endothelium bound lymphocytes were evenly distributed all through the pathological tissue though rarely reaching close to fully developed Reed Sternberg cells (Fig 4, 5, 6) Instead they were often seen in close contact with intraparenchymal lymph tracts, due to the absence of a perivenular zone, rich in lymphocytes which normally separates HE venules from adjacent lymph vessels (Fig 6) To an observer familiar with the normal appearance of HE venules, this feature is strikingly unusual

Specimens from the single case labelled nodular sclerosis offered a somewhat different picture No HE venules were found in the nodules which were rich in Reed Sternberg cells and histiocytic cells, but rather poor in lymphocytes In the broad internodular scars, lymphocytes were seen only in a few tiny scattered foci A high power scrutiny of these foci revealed that many of them were in reality HE venules, often with a rich content of endothelium bound lymphocytes inside a thick and sometimes sclerotic basement membrane (Fig 7) Virtually no lymphocytes were seen on the outside of this membrane HE venules were thus, surprisingly, present also in this case as an *ultimum moriens* remnant

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Fig 3 Lymph node tissue rich in HE venules from one of the cases of CLL mentioned in the text (1 μ section May Grunwald Giemsa × 240)

Fig 4 Reed Sternberg cells (b) and HE venule (a) in lymph node from a case labelled lymphocyte predominance (1 μ section silver impregnation according to Movat × 240)

Fig 5 Reed Sternberg cells (b) and HE venule (a) from a case labelled mixed cellularity (1 μ section toluidine blue × 240)

of organized lymph node tissue in the inter nodular scars

DISCUSSION

In the concept of *Gowans & al* (1964) the HE venules serve the immigration of blood lymphocytes to the lymph node parenchyma This does not explain in full the striking pictures of 'intra endothelial' lymphocytes typical of these vessels which have provoked several fanciful hypotheses in the past One of them was presented by *Vincent & Gunz* (1970) who suggested that HE venules might be implicated in a homeostatic regulation of the number of circulating lymphocytes The high endothelium cells were suggested to recognize immigrating normal lymphocytes on specific surface sites and to induce an adaptation of the release of cells from the lymph node to the "recorded number of immigrants It was a point in the hypothesis that the endothelium cells might fail to detect inflowing *leukaemic* lymphocytes and thus tolerate an excess outflow of lymphocytes in CLL

The original scope of the present study was to test a personal impress on that HE venules are absent in CLL nodes and thus unable to functionate according to the hypothesis of *Vincent & Gunz* (1970) In most of our CLL nodes we could really confirm the absence of HE venules but the histological picture in the series was not uniform and in some cases HE venules proved to be present and even abundant A final evaluation of these findings has to await the analysis of a much larger series but we were impressed by the possibility to discriminate between the structure of diffuse cortex and that of lymphocytic lymphoma by the complete absence of HE venules in the lymphoma tissue

This made us look for HE venules also in non leukaemic types of malignant lymphomas and we cou —as expected—state that HE venules are a ent in the genuine tumour tissue of *lym cytic and lymphoblastic lymphomas*

Instead, HE venu proved to be constantly present (and sometimes even abundant) in the specific tissue of the *Hodgkin* nodes In specimens labelled lymphocyte predominance and mixed cellularity this made the Hodgkin tissue appear, not as an invading lymphoma but rather as a transformed genuine lymph node tissue, structurally akin to the paracortical zone in which the leading structural element, the HE venules had remained virtually unmolested Interesting was the presence of HE venules in the internodal scars of the single case of nodular sclerosis We would like to stress the complete absence of a perivenular cuff of lymphocytes as a specific feature of HE venules in the Hodgkin nodes

A severe depression of cell bound immunity has long been regarded as typical of Hodgkin s disease and may thus be expected to interfer with the activities of *T lymphocytes* A strategic point of such an interference would have been at the level of HE venules the assumed immigration ports for recirculating lymphocytes, an absence of HE venules in Hodgkin tissue as in other lymphomas would have been an attractive explanation of this immune failure From this viewpoint the abundance of HE venules in some Hodgkin nodes may appear surprising It must in this connection be stressed, however that the b o logic function of HE venules is still far from settled (*Sainte Marie & al* 1967), it has even been doubted that the depression of cell bound immunity is a specific feature of Hodgkin s disease (*Young & al* 1972)

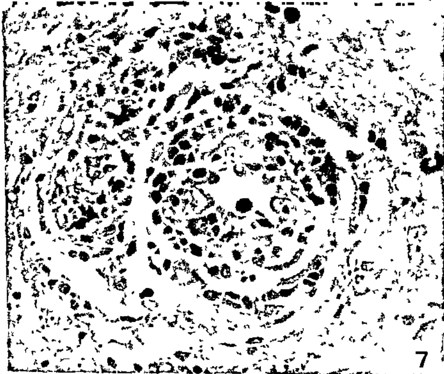
As to the significance of lymphocyte endothelium relat ons in HE venules it has recent

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Fig 6 Lymph node from a case of Hodkin s d sea e classed mixed cellularity A Reed Sternberg cell at b an intraparenchymal lymph s nus at a Arrows point at four HE venules bulg ng d rectly into the s nus lumen without the normal interposition of a perivenular cuff of lymphocytes (1 μ section silver impregnation accord ng to Movat × 240)

Fig 7 HE venules from the scar tissue in a case labelled nodular scleros s No lymphocytes are seen outs de the basement membrane which is cont nu ous with the surro nding scar tissue (2 μ section May Grunwald Giemsa × 240)

6



8

9

Fig 8 HE venules from tonsillar tissue ("chronic tonsillitis) Arrow at d marks the direction of a lymphocyte moving out of the venule through the basement membrane At c typical cluster of 'intra-endothelial' lymphocytes (1 μ section, toluidine blue, × 720)

Fig 9 Detail of tissue culture from tonsillar tissue, showing emperipolesis of lymphocytes with fibroblastlike cells as target cells The lymphocyte clusters at c closely similar to corresponding clusters in HE venules (Phase contrast, × 640)

ly been shown that lymphocytes never lodge *within* the endothelium cells but rather in niche like invaginations of the cell membrane (*Claesson* & *al* 1971, *Schoefl* 1972, *Messier* & *al* 1972) This discussion has many points in common with the previous argumentation regarding the presumed intracellular position of lymphocytes during *emperipolesis* in cell culture (Fig 9), which for good reasons may be regarded as an *in vitro* recapitulation of the lymphocyte-endothelium relations in HE venules (Fig 8) In both cases it is easy to show that lymphocytes never penetrate the cell membrane, but nevertheless are very intimately engaged with their target cells Nobody would pretend that emperipolesis lymphocytes *in vitro* are aiming at anything *beyond* the target cell on the glass of the culture chamber In analogy it is hard to avoid the conclusion that the endothelium cell itself (and nothing beyond it) is the target for the lymphocytes in HE venules, in reality, evidence is still scanty for the assumption that these lymphocytes are purposeful immigrants to the lymph node parenchyma It should not be forgotten that post-capillary venules, in any tissue, represent the vascular segment where tissue fluid is following back to blood, in lymph node presumably carrying i a the IgG demonstrated by *Sordat* & *al* (1971) on the surface of the high endothelium cells

These aspects of the activities of lymphocytes in HE venules appear natural in some of our Hodgkin specimens in which no lymphocytes were present outside the venule and in which the lymphocytes inside the basement membrane appeared to be engaged with the high endothelium cells and nothing else

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Acta path microbiol scand Section A 82, 80-92, 1974

SKIN GRAFTS IN *NUDE* MICE

I *Allografts in* Nude *Mice of Three Genetic Backgrounds (BALB/c, C3H, C57/BL)*

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Mice, homozygous for the *nu* gene (*nude* mice), have no capacity for rejection of allografts as evidenced by this study of nine different graft host combinations The mice concerned stem from an intermediate stage in a *nu* gene transfer to three inbred strains (BALB/c, C3H, C57/BL) Mice of each of the three genetic backgrounds accepted skin grafts from three inbred donor strains, differing at the H 2 locus A preliminary comparison with a concurrent study of heterografts (rat skin) indicates that allografts are not so readily incorporated, and are more prone to endogenous damage during the take process This paradoxical observation is discussed at greater length in the subsequent article on heterotransplants in *nude* mice After allografting, *nude* mice display lymphocytosis and there is lymphoid cell proliferation in the primary follicles of lymph nodes, and increased numbers of mononuclear cells are seen in the paracortex of lymph nodes and in the red pulp of the spleen No cytotoxic alloantibodies could be demonstrated in serum from the graft bearing *nude* mice Thymus grafting confers capacity for allograft rejection

Mice with *nu* gene homozygosity are born without a thymus as first shown by *Pantelouris* (1968) Many studies have subsequently shown that this defect involves profound change in the immune responses of the animal, both cell-mediated (*Rygaard* 1969, *Rygaard & Povlsen* 1969, *Povlsen & Rygaard* 1971, 1972, *Povlsen & al* 1973, *Wortis* 1971, *Kindred* 1971) and humoral as reflected by formation of circulating antibody in response to a variety of antigen challenges Most studied are the effects of sheep red blood cell immunization (*Rygaard* 1969, *Kindred* 1971, *Kindred & Weiler* 1972 *Wortis* 1971, *Pantelouris & Flisch* 1972 *Pritchard & al* 1973)

Rygaard & Povlsen (1974) have shown general uniformity of haematological and morphological changes in *nude* mice irrespective of the animals genetic backgrounds which so far as regards the cited article, were BALB/c, C3H and C57/BL/6 The study was made at an intermediate stage (*Snell* 1948, *Green & Doolittle* 1963) of *nu* gene transfer to the respective strains The known genetic background comprised between 87 and 94 per cent of the genome The continuing study concerns immune responses and genetic background Transplantation studies and studies of plaque forming cells (PFC *Jerne & Nordin*, 1963), were undertaken in animals of the same derivation as above to determine, primarily, whether immune responses were also independent of genetic background

The following concerns allotransplantation of normal skin Subsequent publications will

Received 18 vii 73 Accepted 18 vii 73

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report the results of heterotransplant studies and of *Jerne* plaque assays

A number of previous studies have been made of allotransplantation in *nude* mice of different genetic constitutions (*Wortis* 1971, *Pennycuik* 1971, *Kindred* 1971, *Pantelouris* 1971) Herein are reported the outcomes of a series of recipient—donor combinations involving first and second set allografts Grafts were studied macroscopically and histologically peripheral blood white cell and differential counts were made spleens and lymph nodes were studied macroscopically and histologically in some animals the effects of thymus grafting on iso and allogeneic trans plants were observed, and, finally, serum from a number of the animals was studied for the detection of cytotoxic antibodies

TABLE 1 *Effects of Allotransplantation, Day 7 Fate of Graft Appearance of Lymph Nodes Spleen Weight, WBC*

Recipient Probable H 2 type	Donor (H 2)	No	Accept of graft	Lymph Nodes	Spleen Weight mg	Leuco/µl	Lymph per cent	Lymph/µl
BALB/c, H 2^d	A/J	5832	+	+ +	102	3450	51	1760
	(a)	—33	+	0	71	6580	82	5400
	C3H	6001	+	+	135	4670	76	3550
	(k)	—02	+	+	151	10700	63	6740
	C57/BL	6013	+	+	115	5490	57	3130
	(b)	—14	+	+ +	119	4560	61	2780
	Mean values of BALB/c *nude*				116	5908	65	3893
	S E in brackets				(25)	(2345)	(11)	(1677)
	Reference values*				110	2380	55	1246
					(33)	(641)		(303)
C3H H 2^k	A/J	5844	+	0	138	6590	29	1910
	(a)	—45	+	0	121	5220	71	3710
	BALB/c	5906	+	+	119	6080	54	3280
	(d)	—07	+	0	103	5400	69	3730
	C57/BL	6025	+	0	123	—	70	—
	(b)	—26	+	+	43	2970	27	800
	Mean values of C3H *nude*				108	5252	53	2686
	S E in brackets				(31)	(1241)	(19)	(1153)
	Reference values*				122	2248	57	1294
					(21)	(629)		(475)
C57/BL H 2^b	A/J	5856	+	0	106	4780	77	3680
	(a)	—57	+	0	90	7430	80	5940
	BALB/c	5918	+	0	84	7970	76	6060
	(d)	– 19	+	+	100	5830	72	4200
	C3H	6037	+	0	188	3570	83	2960
	(k)	—38	(+)	0	115	6290	69	4340
	Mean values of C57/BL *nude*				114	5978	76	4530
	S E in brackets				(35)	(1496)	(5)	(1130)
	Reference values*				89	2245		1356
					(22)	(1044)	58	(751)

* from Rygaard & Povlsen 1974

Eighteen normal mice graf ed in the same combinations all rejected first set grafts between day 12 and 16

Lymph Nodes 0 not enlarged + = moderately enlarged, + + = strongly enlarged

TABLE 2. *Effects of Allotransplantation, Day 14 Fate of Graft, Appearance of Lymph Nodes, Spleen Weight, WBC*

Recipient Probable H-2 type	Donor (H-2)	No	Accept of graft	Lymph Nodes	Spleen Weight mg	Leuco/µl	Lymph per cent	Lympl
BALB/c, H-2d	A/J	5834	+	0	86	5650	76	429
	(a)	—35	+	0	69	2910	35	103
	C3H	6003	+	+	89	7990	59	471
	(k)	—04	(0)	+ +	94	7160	50	358
	C57/BL	6015	(0)	0	129	1700	63	1070
	(b)	—16	(0)	+	71	3000	32	960
	Mean values of BALB/c *nude* S E in brackets				90 (20)	4740 (2337)	53 (15)	2607 (1621
C3H, H-2k	A/J	5846	+	+	106	5660	39	2210
	(a)	—47	+	+	150	2930	61	1790
	BALB/c	5908	+	0	119	4580	28	1280
	(d)	—09	+	0	71	8880	72	6400
	C57/BL	6027	(+)	+	84	2600	43	1120
	(b)	—28	(+)	+	98	6380	13	830
	Mean values of C3H *nude* S E in brackets				105 (25)	5172 (2138)	43 (20)	2272 (1900)
C57/BL, H-2b	A/J	5858	+	0	97	4460	57	2542
	(a)	—59	+	0	52	3800	32	1220
	BALB/c	5920	+	+ +	121	3740	67	2510
	(d)	—21	+	+	41	5170	44	2280
	C3H	6039	(+)	0	61	3620	47	1700
	(k)	—40	(+)	0	92	6270	63	3950
	Mean values of C57/BL *nude* S E in brackets				77 (28)	4510 (951)	52 (12)	2367 (849)

MATERIAL AND METHODS

Mice

Recipients were seven to eight week old female *nude* mice of genetic background BALB/c/A/BOM$_f$, C3H/Tif/BOM$_f$, and C57/BL/6 J/BOM$_f$ and normal representatives of these three strains The *nude* mice were the products of the fourth and fifth cycle in the *nu* gene transfer, thus of a constitution comprising 87 to 94 per cent of the respective genome

All animals were purchased from Gl Bomholtgaard, DK-8680 Ry, Denmark Mice were kept for one week before use under spf conditions with a personnel barrier at the Pathologisk Anatomisk Institut, Kommunehospitalet, Copenhagen After operation, the animals were similarly housed but without personnel barriers Makrolon®, type II cages were used Bedding was sterile, autoclaved feed pellets and sterile drinking water was provided Each animal was separately housed Husbandry measures are described in detail elsewhere (*Rygaard* & *Friis* 1973)

Thymus transplantation At four weeks of age, subcutaneous whole thymus grafts were made in a number of *nude* mice, all of BALB/c extraction Donors were of the same strain, less than 24 hours old No account was taken of sex

Donors of the skin were all four to five days old of the inbred strains A/J/BOM$_f$ BALB/c/A/BOM$_f$, C3H/Tif/BOM$_f$, and C57/BL/6 J/BOM$_f$ Sex of donors was not determined These animals were also purchased from Gl Bomholtgaard, and were delivered on the morning of the day they were to be used

TABLE 3 *Effects of Allotransplantation, Day 30 Fate of Graft, Appearance of Lymph Nodes, Spleen Weight, WBC*

Recipient Probable H-2 type	Donor (H 2)	No	Accept of graft	Lymph Nodes	Spleen Weight mg	Leuco/μl	Lymph per cent	Lymph/μl
BALB/c, H-2d	A/J	5836	+	+	165	6340	61	3870
	(a)	—37	+	0	111	3100	68	2110
	C3H	6005	(+)	0	116	5740	41	2350
	(k)	—06	(+)	0	43	4360	29	1260
	C57/BL	6017	(0)	0	126	6000	74	4440
	(b)	—18	(+)	0	107	5650	77	4350
	Mean values of BALB/c *nude*				111	5198	58	3063
	S E in brackets				(36)	(1122)	(18)	(1216)
C3H, H-2k	A/J	5849	+	0	145	2450	54	1320
	(a)	—50	+	0	131	4740	56	2650
	BALB/c	5910	+	0	83	4600	63	2900
	(d)	—11	+	0	138	8800	74	6510
	C57/BL	6029	+	0	96	4570	66	3020
	(b)	—30	+	0	70	3770	69	2600
	Mean values of C3H *nude*				111	4822	64	3167
	S E in brackets				(29)	(1944)	(7)	(1595)
C57/BL, H 2b	A/J	5860	+	0	80	5340	73	3900
	(a)	—61	+	0	142	2760	63	1740
	BALB/c	5922	+	+	100	3840	80	3070
	(d)	—23	+	+	94	3550	68	2410
	C3H	6041	+	+	103	4980	67	3340
	(k)	—42	+	0	79	2970	78	2320
	Mean values of C57/BL *nude*				100	3907	72	2797
	S E in brackets				(21)	(960)	(6)	(717)

EXPERIMENTAL PLANNING

Allografts in Not Prepared Nude *Recipients*

First set grafts were made in three series Each series comprised 6 *nude* mice of each of the three genetic backgrounds, arranged in nine recipient donor combinations, each performed in duplicate Series 1, 2 and 3 animals were killed on days 7, 14 and 30, respectively, after transplantation

Combinations are shown in Tables 1, 2 and 3

Second set grafts were made in a further 24 animals One died during the second operation The remaining 23 received second set grafts, 35 days after the first set, derived from donors of the same strain Animals were observed until natural death, or killed 152 days after first set grafting (117 days after second set grafting) The combinations are shown in Table 4

Allografts in Normal Control Mice

First and second set grafts Control mice were seven to eight week old females from each of the three inbred strains—two animals in each of the nine combinations (as in Table 1 to 3) After first set grafting, *animals were observed until* day 35, whereupon second set grafts were made, the donors being of the same strain as that used for the first set transplant All animals were killed 152 days after the first set graft (117 days after the second set graft)

Isografts in Not Prepared Nude *Recipients*

Two series of studies were made, each comprising eight *nude* mice and four normal mice of BALB/c and C3H strains Donor animals were *four to five day* old normal representatives of the

BALB/c and C3H strains, no account being taken of sex These animals were observed, and killed on day 67.

The Effects of Preparation by Thymus Grafting on Allografts and Isografts

Twenty *nude* mice of BALB/c background received thymus transplants at the age of four weeks as described earlier Four weeks later, 12 of these animals received allografts, five from C57/BL donors, and seven from A/J donors These donor animals were all four to five days old The remaining eight *nude* mice received isografts at the same time from BALB/c donors of similar age, and of either sex at random

Observation

The animals were scrutinized daily Notes were made on the appearance of grafts signs of vascularization, healing at the edges, swelling (oedema), colour, hair growth, signs of trauma, and signs of rejection

Killing of animals Animals were killed by exsanguination through the axillary vessels while under light ether anæsthesia (see *Rygaard & Poulsen* 1974) Blood was taken at this time for haematological study *q v*, and organs and tissues taken at autopsy were prepared for histological study *q v*

Haematology

Blood was taken from the not prepared *nude* recipients of first set allografts for white blood cell counts, and permanent preparations were made for differential counts The actual counts were made in Neubauer's counting chambers after dilution and staining of cells Slides for differential counts were stained with May Grunwald-Giemsa preparation

The remaining blood in these and the other animals was collected in dry tubes Serum was drawn off after centrifuging and stored in Sanz tubes at $-20°C$

Cytotoxicity tests were made during a study tour in the Dept of Tumour Biology, Karolinska Institutet Stockholm, Sweden The tests were made with lymphocytes from the respective donor strains using a modification of the *Gorer & O'Gorman* method (1956)

Autopsy and Histological Studies

After the killing or spontaneous death of animals, autopsies were conducted particularly orientated towards study of the grafts, spleen, lymph nodes, and the identification of any pathological changes

Bodies were fixed as units in Lillie's fluid after making transverse incisions in the skin of the back 5 mm cranial and 5 mm caudal to the graft(s) Following the fixation the graft and adjacent tissues, including an extensive dissection of the back and spine to ensure best preservation of the graft bed, were removed together Laterally the resection included 5-6 mm of recipient skin which allowed study of the graft/recipient skin transitional zone After decalcification (Rapid Decalcifier Bethlehem Trading, Gothenburg, Sweden), the tissues—graft sections, spleen and lymph nodes—were embedded in paraffin wax and 7 µm sections were taken These sections were stained, a.m van Gieson Hansen, with hæmatoxyline and eosine and methyl green pyronine (*Pearse* 1960)

Grafting Technique

Propanidum (Epontol®, Bayer AG, BRD) 0 5 mg/g BW was administered to recipients intraperitoneally, and the animals were laid on sterile cloths on their stomachs with no form of restraint. 12×12 mm sections of skin were removed from the back, centred at about the 10th thoracic vertebra, more cranial if the animals were destined for second set grafts The thus prepared graft beds were covered with sterile gauze Bleeding was, as a rule, minimal Donors were placed in a refrigerator at $+4°$ C for approximately 10 minutes, and, thereafter, decapitated high up the neck Two grafts can be obtained from the back of each donor The free skin graft is turned over a finger tip and scraped clean of loose connective tissue and muscle with the sharp blade of a fine pair of straight scissors. Care is taken to free the dermis completely from underlying tissues, while avoiding perforation The graft is then immediately set in the graft bed With experience, it is practically always possible to achieve continuous contact of graft edge and recipient skin edge If a graft is too large it is cut to size, if too small, it is discarded Immediately on completion of matching graft with graft bed the graft is fixed in place with Histoacryl® (Braun Melsungen, BRD), an absorbable neutral tissue adhesive No bandages or muzzles are used Recipients remain anæsthetized for approximately seven minutes, which allows ample time for the procedure They are observed until spontaneous walking movements begin, and then placed in individual Makrolon® type II cages To obviate risk of aspiration, the bottoms of cages are covered with paper napkins for the first day, whereafter these are replaced by the usual wood granulate Animals are kept in individual cages over the whole of the observation period to avoid trauma to grafts which might occur during combat

RESULTS

Observation and Macroscopic Studies

Allografting in not-prepared nude *recipients* Outcomes were virtually uniform irre-

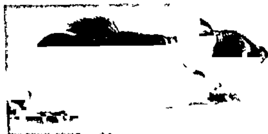


Fig 1 Nude mouse of C57/BL background, with accepted C3H skin graft Normal hair growth Day 30



Fig 2 Nude mouse of BALB/c background with accepted A/J skin graft Sparse hair growth Day 30

spective of the genetic background of animals, and irrespective of the recipient donor combination After two days, the graft is oedematous, but there are already signs of vascularization—spread, darker red, spider-like structures which give the graft a hyperaemic appearance This change is seen first at the margins, while centrally there is often a scabbed appearance Small areas of the graft may be dry, but actual necrosis and ulceration do not occur About days 5 & 6 oedema is resolved in some areas but persists in others The pattern is not constant Scabs separate between the 10th & 12th day, and the underlying graft frequently appears to be atrophic, but macroscopically vital About day 14, hair growth is clearly evident There is large variation in its distribution There may be pockets of tufted growth interspersed by areas of no, or sparse, growth, or there may be luxuriant growth over the whole of the graft, the colour being that of the donor mouse This hair growth continues up to the 30th day, and is normal and full in some animals, but remains stunted and ab normal in distribution in others These appearances were maintained in the longest observed animals—those receiving second set grafts Moderate to severe graft contraction was observed on occasion, but these grafts remained as otherwise described The results of these grafting experiments are collected in Tables 1, 2 & 3 (Fig 1–6)

In the 18 *nude* recipients killed on day 7, grafts were intact in 17 while there was some doubt in the remaining animal

On day 14, 11 grafts were clearly accepted, four probably, while in three there was suspicion of rejection These latter three *nudes* were all of BALB/c origin one graft was from the C3H strain, and the other two from C57/BL

On day 30, 14 grafts were certainly accepted, three probably, while in one there was suspicion of rejection This animal was also of BALB/c origin, as, in fact, were all recipients where graft take could only be de scribed as probable

In the 24 *nude* mice recipients intended for second set grafts, all first set grafts were macroscopically accepted There was hair growth and no erythema or ulceration

Thus, at days 30 to 35, 38 of 42 recipients had certainly accepted the grafts, and three probably had Only in the one instance was there real doubt

Second Set Grafts

As recorded, one of the 24 animals died during the second set graft operation The other 23 were observed until spontaneous death (Table 4), or until killed on day 152 Eight of the 23 lived to be killed Fate of second set grafts was similar to that of first set One animal died on day 81 after second set graft ing, and there were signs of probable rejection The remaining 22 animals were hosts of healthy first and second set grafts at the time of spontaneous death or sacrifice As mentioned, maximal observation was made in the eight animals killed on day 117 after

TABLE 4 *Results of First and Second Set Allotransplantation Fate of Grafts and Duration of Observation*

Recipient Probable H 2 type	Donor (H 2)	No	Accept		Observation Days	
			I	II	I	II
BALB/c, H 2d	A/J (a)	5838	+	+	74	39
		—39	+	+	90	55
		—40	+	0	116	81
	C3H (k)	6009	+	+	152	117
		—10	+	+	152	117
	C57/BL (b)	6021	+	+	56	21
		—22	+	+	44	9
C3H, H 2k	A/J (a)	5851	+	+	63	28
		—52	+	+	91	56
		—53	+	+	71	36
	BALB/c (d)	5912	+	+	101	66
		—13	+	+	69	34
		—14	+	n d	35	—
		—15	+	+	152	117
	C57/BL (b)	6031	+	+	152	117
		—32	+	+	69	34
		—33	+	+	46	11
C57/BL, H 2b	A/J (a)	5862	+	+	152	117
		—65	+	+	40	5
	BALB/c (d)	5926	+	+	91	56
	C3H (k)	6043	+	+	152	117
		—44	+	+	93	58
		—45	+	+	152	117
		—46	+	+	152	117

Recipients surviving for 152/117 days were sacrificed for investigation All other recipients died spontaneously
Eighteen normal mice second set grafted in the same combinations, all rejected the grafts Killed for investigation on day 152/117

second set grafting Their first set grafts were thus followed for 152 days

Allografting in Normal Recipients (First and Second Set)

Grafts were rejected in the 18 control animals and shed between the 12th and 16th days Appearances up to this time were of spreading necrosis with no signs of hair growth Second set grafts were similarly, but more rapidly, rejected Graft beds were rapidly re-epithelialized, so that on sacrifice 32 days after second set grafting it was not always possible to define the beds

Isografts in Not Prepared Nude *Recipients*

Both *nude* and normal recipients (BALB/c and C3H) accepted the isografts In two of the BALB/c *nude* recipients, grafts evidenced some contraction Hair growth was good in all animals (Table 5)

TABLE 5 *Fate of Isografts and Allografts in Not Prepared and Thymus Grafted Nude Mice, and Normal Inbred Mice*

Recipients	Fate of Isografts	Fate of Allografts
20 BALB/c *nude* mice prepared by thymus grafting 4 weeks before skin grafting	8 BALB/c grafts accepted	5 C57/BL grafts rejected 7 A/J grafts rejected
8 not prepared BALB/c *nude* 4 BALB/c normal mice 8 not prepared C3H *nude* 4 C3H normal mice	All grafts accepted	

All recipients observed until day 67

Allografts and Isografts in Nude *Mice with Thymus Transplants*

The 12 allografts (five from C57/BL, and seven from A/J donors) were all rejected and shed within 14 days The eight isografts were all accepted,—Table 5

Macroscopic Findings at Autopsy

Spleen and lymph nodes Spleen weights in not prepared *nude* mice are given in Tables 1, 2 and 3, covering the period up to and including day 30 For comparison, mean spleen weights of not prepared and not transplanted *nudes* of similar backgrounds (*Rygaard* & *Povlsen* 1974) are appended It is apparent that there is a *temporary* increase in spleen weight in the transplanted animals of the C57 genetic background This was also the immediate visual impression at autopsy

The appearance and size of lymph nodes are also noted in the same tables

Histology

Allografts in not prepared nude *recipients Day 7* Grafts were markedly oedematous, and there was a mild to moderate granulocyte and histiocyte infiltration In some cases epidermiolysis and re epithelialization was seen, (cfr day 14) Below the grafts a very marked fibroblast proliferation was a constant finding (Fig 3–6)

Lymph nodes displayed enlarged primary follicles which usually appeared as a confluent subcapsular rim Number of mononuclear cells in the paracortical region (inner cortex) was increased, and diapedesis in the postcapillary venules was marked Aggregations of mononuclear cells were seen in the medullary cords, and frequently a number of large pyroninophilic cells There were no secondary follicles (Fig 9)

In the spleen increased numbers of mononuclear (lymphoid) cells were apparent in small clumps in the red pulp

Day 14 Graft oedema much reduced, as were the numbers of fibroblasts in the graft bed When graft acceptance was in doubt, characteristic changes were seen there were necrotic areas in the graft of greater or lesser extent, associated with a separation of the graft dermis superficially, or deeper at about the mid point of hair follicles Above the separation the superficial layer of the graft was clearly in the process of shedding but below, the deeper aspect of the hair follicles with adjacent dermal connective tissue was vital, and there was extensive re epithelialization At the edges, the origin of the new epithelium could not be determined, but might be of host origin Centrally, it appeared that the new epithelium was derived from the epithelium of the hair follicles (Fig 6) In some of the sections there was also spread necrosis of the deeper graft layers

Lymph nodes and spleen were of the same appearance as on day 7

Day 30 At this stage the graft host relationship was stable Roughly speaking, two groupings could be made a) total graft ac-

Fig 3





ig 4



F



ig 7



ig 8



Fig



ceptance with uniform, normally developed hair follicles and dermis, and no inflammatory changes, or b) total acceptance, but grafts affected during the course after plac ing The dermis of these grafts was flatter, and there were areas of hair follicle loss or degress of atrophy No inflammatory changes were evident (Fig 7, 8)

The appearances of lymph nodes and spleen were not further altered, apart from the finding of small secondary follicles in the lymph nodes of three animals

Second set grafts, day 117 up to day 152, were of the two appearances described for day 30

Allografts in normal control mice, both first and second set, were rejected, and histological studies were not made

Isografts in not-prepared nude *recipients* *and normal mice* were well macroscopically accepted with good, uniform hair growth Histological studies were not made

Nude, *thymus grafted recipients* No histological studies were made

Fig 3 Transverse section of *nude* mouse of BALB/c background carrying C57/BL skin graft Day 7 12 ×, H&E

Fig 4 Detail of (3), showing transition between host skin (left) and donor skin (right) Prolifera tion of fibrous connective tissue and cellular infil tration 32 ×, H&E

Fig 5 Detail of (3), showing proliferation of fi brous connective tissue and cellular proliferation between graft and muscular layer of host Necrosis and lysis of epidermis and superficial dermal tissue and re-epithelialization 80 ×, H&E

Fig 6 Detail of central part of (5), showing re epithelialization from epithelium of hair follicles 200 × H&E

Fig 7 *Nude* mouse of BALB/c background, carry ing C3H skin graft Graft contracted Atrophy of hair follicles Pronounced proliferation of fibrous connective tissue with sparse cellular infiltration Day 30 32 ×, H&E

Fig 8 *Nude* mouse of C57/BL background, carry ing A/J skin graft Transition between host skin (left), and donor skin (right) Graft well accepted with well developed hair follicles No cellular in filtration Day 30 32 ×, H&E

Fig 9 Part of lymph node of C3H *nude*, carrying BALB/c skin graft day 7 Hypertroph c primary follicle in outer cortex (right), lymphocytic infi tration in adjacent paracortical area and in medul lary cords 125 × H&E

Haematology

Total white blood cell counts, the percentage of lymphocytes, and the absolute lymphocyte counts per μl are given in Tables 1–3 Comparable findings in not transplanted *nudes* of similar stock are appended The transplanted animals display both leuco and lymphocytosis

Serum Cytotoxicity Studies

The findings are recorded in Table 6 The normal control mice with allografts had markedly cytotoxic serum, while no cytotoxic antibodies could be demonstrated in the not-prepared allografted *nude* mice

DISCUSSION

As apparent from the recorded results, there is a predominant tendency in *nude* mice, irrespective of genetic background and donor type, to accept allografts Collecting the long term results, those in animals after 30 to 35 days of observation, grafts were clearly accepted in 38 of 42 animals, and probably accepted in three of the four re maining One graft only was rejected and shed These evaluations stem from macrosco pic and microscopic studies, and agree well with others' findings (*Wortis* 1971, *Pennycuik* 1971, *Kindred* 1971, *Pantelouris* 1971)

Second set grafts of similar origin to the first set were also accepted in 22 of 23 *nude* recipients Fifteen of these animals died during the course of the study at the end of their normal life spans–four to five months–with the usual signs of wasting but the re maining eight survived until sacrifice at seven months The animals evidenced full acceptance of grafts placed four and five months previously

In further evaluation of these results, how-

TABLE 6 *Cytotoxic Indices of Sera from Nude and Normal Mice after Allotransplantation*

Recipient Strain/Back ground Strain	Donor	Grafted *Nude* C I	Grafted Normal C I	Untreated *Nude* C I	Untreated Normal C I
BALB/c	A/J	0 00	0 50	0 00	0 00
		0 00	0 40	0 00	
	C3H	0 00	0 70	0 00	0 00
		0 01	0 95	0 00	
	C57/BL	0 00	0 00	0 00	0 00
		0 00	0 00	0 00	0 00
C3H	A/J	0 00	1 00	0 00	0 00
		0 00	0 35	0 00	
	BALB/c	0 0?	0 25	0 00	0 00
		0 02	0 45	0 00	
	C57/BL	0 00	1 00	0 00	0 00
		0 00	0 10	0 00	
C57/BL	A/J	0 00	1 00	0 00	0 00
		0 00	1 00	0 00	
	BALB/c	0 01	0 20	0 00	0 00
		0 02	—	0 00	
	C3H	0 04	0 65	0 00	0 00
		0 02	0 60	0 00	

Nude and normal sera tested in titre 1 2 and 1 4 respectively against lymphocytes from donor strain mice
First set grafted *nude* mice killed on day 30
First and second set grafted normal mice killed on day 152/117 (Cfr Tables 3 & 4)

ever it is necessary to make comparison with similar studies in *nudes* of the same three backgrounds which were recipients of rat skin heterotransplants (*Rygaard* 1974)

The *allotransplants* were observed macroscopically and histologically over the first two weeks to excite considerable host reaction in the form of oedema proliferation of fibrous connective tissue and cellular infiltration in the graft and region of the graft A superficial necros s and shedding of the superficial graft layer was also frequently observed while the acceptance process was mediated at deeper graft level with the re epithelialization probably stemming from the epithelium of the middle part of the hair follicles The consequences of this disturbed process were evident in the often delayed and impaired hair growth which could be stunted or tufted and interspersed with bare atrophic — although vital—areas of graft tissue This was confirmed by histological evidence of epidermal and hair follicle atrophy and of dermal connective tissue infiltration There was also a tendency to graft contraction

The *heterotransplants*, by contrast were much more rapidly and certainly accepted the only initial reservations being dictated by a transient central scab formation which was rapidly shed to reveal normal graft tissue The end result in all instances was a taken graft with tight normal fur growth Infrequently, slight graft contraction was observed The histological findings resembled to some extent those in animals with allotransplants but the changes were always less marked and more rapidly resolved Acceptance of heterografts, as evaluated from time from hair follicle development and function and from time of resolution of macroscop cally observed reaction is qualitatively much superior to acceptance of allografts The possible im

plications of this recording are discussed in the subsequent article on heterografts in the *nude* (*Rygaard* 1974)

The reaction against allografts by *nude* recipients is also evidenced by changes in spleen and lymph nodes Lymph node enlargement was modest, but characteristic qualitative changes were apparent—increase in size of the primary follicles, and increased numbers of lymphocytes in the thymus dependent paracortical area Small aggregations of lymphoid cells also appeared in the spleen, primarily in the red pulp, but also occasionally around the arterioles of the Malpighian bodies,—that is to say, also in the thymus dependent region of the spleen A study of the origin and kinetics of these cells would be of some interest

Isografts were freely accepted by both not-prepared and thymus transplanted *nude* mice, as by inbred mice of the basic strains A comparative study of the early course after allografting and isografting in nude mice would be of value in separating non specific, operatively induced inflammatory reactions from possibly specific reactions of other nature Such a study would have to be made in *nudes* in which gene transfer was complete

Preparation of *nudes* by thymus transplantation entails that allografts are rejected as by normal recipients, which is as reported by others (*Kindred* 1971, *Pantelouris* 1972) The haematological findings of leucocytosis, particularly a lymphocytosis persistent after other parameters had returned to normal after graft acceptance, were also apparent in the heterotransplant (rat to mouse) studies

Finally, and of special interest, the cytotoxicity tests did not detect cytotoxic antibodies in the serum of the 18 *nude* recipients of primary allografts which were sacrificed on day 30 By contrast, moderate to high cytotoxic indices were found in 16 of 18 first and second set grafted normal mice of the three inbred strains It would appear that the *nude* mouse cannot form cytotoxic alloantibodies Heterotransplants, however rat skin (*Rygaard* 1974) and Burkitt s lymphoma tissue (*Poulsen et al* 1973), stimulate cytotoxic heteroantibody production in the *nude*

The allotransplants in normal recipients provoked cytotoxic antibody production, and this evidences that the grafts used in all animals—from four to five day old donors—are "developed from the antigenic point of view This is also made apparent by the work of *Patthey & Edidin* (1973) These authors report evidence of H 2 antigenicity in 8 day old mouse embryos

Neonatal donors are particularly well suited for use in *nude* transplant studies Shaving of grafts is unnecessary, and inspection of early changes is facilitated by the thinness of the skin Thus, early signs of vascularization and rejection are more readily apparent, and, physically, recipient and donor skin is better matched

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The expert technical assistance of Miss *Inger Bull*, and Mr *Oluf Rasmussen s* constant care for the animals is gratefully acknowledged

I wish to thank Dr *Eva Klein* and Miss *Susanne Becker* for valuable advice and assistance during my stay in the Department of Tumor B ology Karolinska Institutet Stockholm Sweden

The study was supported by *The Danish Cancer Society* and the *Danish Medical Research Council*

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SKIN GRAFTS IN *NUDE* MICE

2 *Rat Skin Grafts in* Nude *Mice of Three Genetic Backgrounds (BALB/c, C3H, C57/BL)* *The Effects after Preparation by Thymus Grafts*

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Nude mice of each of the three genetic backgrounds BALB/c, C3H, and C57/BL readily accept skin heterotransplants from inbred Fischer strain rats and other rat strains Second set transplants from the same donors are also readily accepted In all instances graft take was uneventful and the final appearance of grafts was as in normal animals of the donor strain This event contrasts strongly with that observed in similar *nude* mice receiving allotransplants Allograft take was delayed and although ultimate acceptance was virtually 100 per cent, many grafts evidenced endogenous damage occurring during the acceptance process This damage was manifested by tufted irregular hair growth in many cases, and by bare atrophic, though vital, areas of graft skin Also strikingly contrasting were the findings that allografting in *nude* mice excited no cytotoxic antibody response, while cytotoxic anti body titres in serum of *nude* mice receiving heterotransplants were moderate to high. Thymus grafting confers capacity for heterograft rejection

An earlier work (*Rygaard* & *Povlsen* 1974) studied the effects of *nu* gene homozygo sity in mice of three distinct genetic backgrounds (BALB/c, C3H, C57/BL) as reflected in peripheral blood elements, serum proteins, and the histological structure of lymphoid and haemopoietic tissues There was uniformity in these effects, irrespective of the genetic background of the animals

Further comparative studies have been made of the homozygous *nude* mice and mice of the inbred strains to which the *nu* gene was transferred These studies concern a variety of transplantation experiments, and a series of 'in vitro studies of haemolytic plaque formation (*Jerne* & *Nordin* 1963)

Received 22 vii 73 Accepted 22 vii 73

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This particular report concerns heterotransplantation in the mice, the donor animal being the rat After transplantation the animals were observed, and histological and haematological studies were made on the sixth, tenth and thirtieth day Second set transplantations were made on the thirtieth day, and observation was continued for a further 30 days

In some *nude* mice of BALB/c derivation, neonatal thymus tissue derived from donors of the respective inbred strain was trans planted The effects of this on the rat trans plants were observed Finally, some studies of cytotoxic antibodies were made

An earlier report (*Rygaard* 1969) also concerns rat skin transplantation to *nude* mice, and this present is an extension of the preliminary report, but additionally con cerned with the effects of the *nu* gene on

transplants in animals of different genetic backgrounds Parallel comparative studies were made in the respective normal animals

MATERIAL AND METHODS

Mice

Nude mice female mice, seven to eight weeks old, of the three genetic backgrounds BALB/c, C3H, and C57, were used The proportion of the respective inbred strain in the *genome constituted* between 87 and 94 *per cent*, an expression that the *nudes derived from the* 4^{th} to 5^{th} cycle in a gene *transfer* The animals were supplied from the Gl Bomholtgaard Breeding Centre, DK 8680 Ry, Denmark For details of the breeding and husbandry *reference should be made to earlier publications* (*Rygaard & Friis* 1973, *Rygaard & Povlsen* 1974)

Reference animals seven to 8 week old female mice of the above mentioned inbred strains obtained from *the same supplier*

Thymus donor mice new born (less than 24 *hours* old) of the BALB/c strain bred at the Pathological Anatomical Institute, Kommunehospitalet, Copenhagen, Denmark

Control serum for the cytotoxicity studies was derived from other untreated inbred mice and *nude* mice

Rats

Donor animals of both sexes of *the inbred* Fischer strain, spf reared, aged from 4 to 5 days were supplied on the day of use by Veterinary surgeon *K E Møllegård*, Ejby, DK 4623 Ll Skensved Denmark

Grafting techniques both for thymus and skin transplants are previously described (*Rygaard* 1974)

Husbandry animals were kept in individual cages at the Pathological *Anatomical Institute*, fed with autoclaved pellets and sterile drinking water, and observed as previously described (*Rygaard* 1974)

Killing and Preparations

The animals were killed at the specified times exsanguinated and preparations made for microscopy as elsewhere described (*Rygaard* 1974) Serum samples were stored at -20° C

Cytotoxicity Stud s

These experim n s were conducted in the Department of Tum Biology Karolinska Institutet Stockholm, Swed during a study tour The method was as use he department, a modification of that rep by *Gorer & O Gorman* (1956)

Study Plan

Five series of transplantations were undertaken

1) Four *nude* mice and two normal mice from each of the three strains were transplanted and killed on day 6 (Table 1)

2) as in 1) but the animals were killed on day 10 (Table 2)

3) Two *nude* mice and one normal mouse from each of *the three strains* were transplanted and killed on day 30 (Table 3)

4) Two *nude* mice and one normal mouse from each of *the three strains* were transplanted on day 0, and received second set transplants on day 30 These animals were killed on day 60 (Table 4) None of the animals in this series 1)–4) received any pre grafting preparation

5) Sixteen *nude* mice, four not prepared and 12 with thymus grafts received rat skin grafts and were killed *on the* 42^{nd} *day* *Thymus grafting* was performed 12 days before skin grafting (Table 5)

The transplantation results, the *appearances* of spleens and lymph nodes and *the peripheral blood* pictures are given in the respective tables The macroscopic and microscopic appearance of grafts and lymphoid tissue are described together under the heading of ' results

RESULTS

The course following grafting was uniform in all the not prepared *nude* mice irrespective of genetic background Over the first two days the graft edges displayed incorporation in the graft bed There was mild oedema and the grafts were pale, but about *the second day* early signs of revascularization became apparent Diffuse petechial like spots appeared over the whole graft surface though most concentrated at the periphery Over the next three to four days graft colour became normal, occasionally somewhat erythematous Yellowish grey scablike formation was sometimes observed predominantly about the centre of the graft These scabs appeared from the fourth day onwards but were shed spontaneously sometime after day 10 revealing graft tissue of normal appearance Oedema which thickened the grafts resolved slowly, but graft thickness was maintained—increased—by the substance increase occasioned by growth of hair follicles and connective tissue, so that the graft was elevated above the level of the adjacent host skin Healing in of the graft edge was usually complete by



Fig 1 & *Fig 2* *Nude* mice of BALB/c background carrying first and first/second set skin grafts from inbred Fischer rat donors Day 30 and 60/30 respectively

day 6 but rarely delayed until day 10 if graft placement had not been smooth Also about the sixth day, marked increase in hair growth was apparent Growth continued between the 14th and 20th day In all recipients close growth of hair up to 15 mm in length was established about day 20 There were no signs of graft contraction (Fig 1)

Normal Recipients

Marked differences between the *nude* mice and normal recipients were apparent on the second day In the latter oedema was much more extensive Signs of vascularization of the graft first appeared around days 5–6 and were diffusely haemorrhagic in character The oedema resolved slowly and red necrosis was manifest between days 10 and 12 Grafts were shed spontaneously few days later

Second Set Grafts

These grafts were treated virtually exactly as described above in the two groups but rejection was accelerated in the normal mice (Fig 2)

Grafts in Thymus Grafted Nude *Mice*

The course after grafting fell between the extremes recounted above In some instances early necrosis began after an initial incorporation and grafts were shed between days 15–20 there never having been any hair growth Otherwise graft take seemed likely with good incorporation and rich hair growth, but about the 30th day erythema and ulceration were manifest and rapid graft shedding followed Two of the animals accepted the grafts but by contrast with the 10 animals which rejected, the thymus graft could not be recovered at post mortem and none of the signs of reconstitution in lymph nodes and spleen associated with successful thymus transplant was apparent (see below) The results of the transplantations in each of the five series of experiments are otherwise recorded in Tables 1–5

Autopsy

Autopsy was undertaken in all animals No pathological changes of any significance in the evaluation of the results were found

TABLE 1 *Effects of Heterotransplantation, Day 6*
Fate of Grafts, Appearance of Spleen and Lymph Nodes, WBC

Strain	Group	No	Graft§ acc	Spleen enlarged	Lymph nodes Lfc$	Lymph nodes GC	Leuco/µl	Lfc %	Lfc/µl
BALB/c	*Nude* mice	6319	+	+	+	0	1660	47	780
		20	+	+	+	+	4070	30	1221
		21	+	+	+	0	4520	53	2396
		22	+	+	+	0	3810	53	2020
		Mean values of BALB/c *nude* (S E in brackets)					3515 (1100)	46 (9)	1604 (638)
		Reference values*					2380 (641)	55	1246 (303)
	Normal	6325	0	0	?	+	6350	79	5017
		26	0	0	?	+ +	6180	70	4326
C3H	*Nude* mice	6310	+	+	+	0	2120	31	660
		11	+	+ +	+ +	0	2250	34	765
		12	+	0	+	0	3820	35	1337
		13	+	+	0	0	2480	42	1042
		Mean values of C3H *nude* (S E in brackets)					2668 (678)	36 (4)	951 (263)
		Reference values*					2248 (629)	57	1294 (475)
	Normal	6316	0	0	0	+	4370	41	1792
		17	0	0	0	+	4170	71	2961
C57/BL/6	*Nude* mice	6301	+	+	+	0	4880	86	4197
		02	+	+	+	0	2440	58	1415
		03	+	+ +	+	0	4140	65	2691
		04	+	0	+	0	3260	58	1891
		Mean values of C57/BL *nude* (S E in brackets)					3680 (917)	67 (11)	2549 (1055)
		Reference values*					2245 (1044)	58	1356 (751)
	Normal	6307	0	0	?	+	4770	78	3721
		08	0	0	?	+	5040	91	4586

§ combined macroscopic and microscopic evaluation
* from Rygaard & Povlsen, 1974
GC germinal centre—secondary nodule
Lfc lymphocytes, $ lymphocytosis of subcapsular and paracortical area.

Fig 3 Transverse section of four day old inbred Fischer rat showing appearance of donor skin at the time of grafting 125 × H&E

Fig 4 Section of Fischer rat skin graft in BALB/c *nude*, day 6 Slight proliferation of fibrous connective tissue (F) in graft bed Note rapid development of hair follicles 125 × H&E

Fig 5 Section of Fischer rat skin graft in normal BALB/c mouse, day 6 Graft totally necrotic Marked haemorrhage (arrow) between graft and infiltrated graft bed 125 ×, H&E

Fig 6 Section of Fischer rat skin graft in BALB/c *nude* recipient, day 30 Hair follicles well developed No reaction against the graft G graft M muscular layer of *nude* recipient 125 ×, H&E








G
M

TABLE 2 *Effects of Heterotransplantation Day 10*
Fate of Grafts, Appearance of Spleen and Lymph Nodes, WBC

		No	Graft§ acc	Spleen enlarged	Lymph nodes Lfc$	Lymph nodes GC	Leuco/µl	Lfc %	Lfc/µl
BALB/c	*Nude* mice	6719	+	+	+	0	4930	37	1825
		20	+	0	+	+	9000	65	5910
		21	+	+	0	0	8560	50	4280
		22	+	+	+	0	3630	46	1670
		Mean values of BALB/c *nude* (S E in brackets)					6553 (2326)	50 (10)	3421 (1771)
	Normal	6725	0	+	+	+	6610	75	4980
		26	0	+	+	+	3870	92	3560
C3H	*Nude* mice	6710	+	0	+	0	3790	74	2805
		11	+	+	0	0	1860	81	1510
		12	+	+	+ +	+	–	70	–
		13	+	+	+	+	2880	50	1440
		Mean values of C3H *nude* (S E in brackets)					2843 (788)	69 (12)	1918 (628)
	Normal	6716	0	+	+	+	6130	92	5610
		17	0	+	+	+	–	94	–
C57/BL/6	*Nude* mice	6701	+	+	+	0	3360	69	2320
		02	+	+	+ +	0	4390	54	2371
		03	+	+	+ +	0	6900	65	4485
		04	+	+	+ +	0	11100	62	6882
		Mean values of C57/BL *nude* (S E in brackets)					6438 (2984)	63 (6)	4015 (1872)
	Normal	6707	0	+	+	+	14900	77	11473
		08	0	+	+	+	10500	94	9870

Explanations same as given in Table 1

Spleens and Lymph Nodes

Macroscopically both *nude* mice and normal mice of each of the three strains evidenced hypertrophy of both spleen and lymph nodes This hypertrophy was moderate and variable Lymph nodes were often cystic which in part explains the increased size

MICROSCOPIC STUDIES

Appearances of Grafts in not Prepared Nude *Mice*

Day 6 graft tissue was always represented in the sections taken for study The graft bed was always clearly identifiable from the lacking panniculus musculorum Healing was complete at the graft edge, the two epithelia blending together The grafts were thickened, mildly oedematous, resting on a layer of proliferating fibrous connective tissue with moderate granulocyte and histiocyte infiltration Very few mononuclear cells were apparent Hair follicles in the graft were at similar developmental stage to that observed in normal rats at the same period of time (Fig 4) For comparison a microphoto of donor rat skin is shown (Fig 3) Occasionally small areas of the superficial epidermis appeared to be loose

Day 10 the main difference from the day 6 study was the striking increase of growth of the graft Well developed hair was apparent in the follicles and dermal connective

TABLE 3 *Effects of Heterotransplantation, Day 30*
Fate of Grafts Appearance of Spleen and Lymph Nodes, WBC

		No	Graft§ acc	Spleen enlarged	Lymph nodes Lfc$	GC	Leuco/μl	Lfc %	Lfc/μl
BALB/c	*Nude* mice	6723	+	0	+	0	4300	94	4042
		24	+	+	+	+	2520	82	2066
		Mean values of BALB/c *nude*					3410	88	3054
	Normal	6727	0	+	+	+	11700	97	11349
C3H	*Nude* mice	6714	+	0	+	0	2990	63	1885
		15	+	0	+	0	4290	96	4118
		Mean values of C3H *nude*					3640	80	3001
	Normal	6718	0	0	+	+	2290	97	2221
C57/BL/6	*Nude* mice	6705	+	0	+	0	7770	72	5595
		06	+	+	+	0	7280	81	5897
		Mean values of C57/BL/6 *nude*					7525	76	5746
	Normal	6709	0	+	+	+	13600	95	12920

Explanations same as given in Table 1

TABLE 4 *Effects of First and Second Set Heterotransplantation Day 60–30 Respectively*
Fat of Grafts Appearance of Spleen and Lymph Nodes, WBC

		No	Grafts§ acc I	II	Spleen enlarged	Lymph nodes Lfc$	GC	Leuco/μl	Lfc %	Lfc/μl
BALB/c	*Nude* mice	6824	+	+	0	+ +	0	5100	47	2397
		25	+	+	+	+ +	0	7650	67	5126
		Mean values of BALB/c *nude*						6375	57	3761
	Normal	6828	0	0	+	+	+	6580	92	6054
C3H	*Nude* mice	6817	+	+	+	+ +	+	1910	50	955
		18	+	+	+	+ +	0	2160	57	1231
		Mean values of C3H *nude*						2035	54	1093
	Normal	6821	0	0	+	+	+	4350	90	3915
C57/BL	*Nude* mice	[illegible]	+	+	+	+	0	–	82	–
		13	+	+	+	+ +	0	9140	80	7312
		Mean values of C57/BL *nude*						9140	81	7312
	Normal	6816	0	0	+	+	+	3960	92	3643

Explanations same as given in Table 1

tissue was much increased By contrast the fibrous connective tissue in the graft bed was very much less evident, and the number of infiltrating cells was much reduced

Day 30 graft acceptance was complete No recipient reaction could be observed at any site Donor and recipient epithelium and donor and recipient dermis blended smoothly

TABLE 5 *Effects of Thymus Grafting on Rat Skin Grafts, Lymph Nodes and Spleen Results on Day 42 after Skin Grafting*

Recipients	Fate of graft	Lymph nodes	Spleen
12 BALB/c *nude* mice prepared by thymus grafting 12 days before skin grafting	8 rejected microscopically day 15 35	Reconstitution of paracortical area Numerous secondary nodules	Reconstitution of periarteriolar area. Numerous secondary nodules
	2 rejected microscopically day 42		
	2 accepted microscopically	No reconstitution	No reconstitution
4 BALB/c *nude* mice not prepared before skin grafting	4 accepted	No reconstitution	No reconstitution

at the junctions Hair development was complete (Fig 6)

Normal Recipients (Fig 5)

Day 6 apparent graft necrosis with marked fibrous tissue proliferation in the graft bed There was marked haemorrhagic reaction at the junction of the two tissues, and the connective tissue was massively infiltrated with granulocytes and mononuclear cells

Day 10 the grafts were now totally necrotic and clearly separated from the graft bed by a massive interposed layer of mononuclear cells

Day 30 all grafts had been shed

Second Set Grafts—not Prepared Nude *Mice*

These animals were preserved for 60 days after first set grafting and received the second set graft at day 30 Both first and second set grafts evidenced complete acceptance as described above for *first set grafts*

Grafts to Thymus Grafted Nude *Recipients*

Day 42 as recorded two of these 12 animals accepted the grafts and at autopsy they appeared exactly as the not thymus grafted *nude* mice Two other animals continued to host small necrotic graft remnants which were completely separated from the graft bed by a massive layer of mononuclear cells The remaining eight animals had shed the grafts and re-epithelialization of the prepared graft beds was complete

HISTOLOGICAL STUDIES OF LYMPHOID TISSUE

Not Prepared Nude *Mice*

Spleen increased numbers of mono nuclear cells were seen in the red pulp No changes were observed in the periarteriolar areas of the Malpighian bodies

Lymph nodes the nodes were enlarged reflecting primarily, an increase in size of the primary follicles which was already apparent on day 6 and persisted over the whole period of the study In 'normal —not transplanted—*nude* mice these follicles constitute a small broken subcapsular rim while in the transplant recipients, the follicles became confluent forming a well demarcated broad subcapsular zone (Fig 7) In isolated instances usually from day 10 onwards secondary follicle formation was seen Numbers of mononuclear cells in the paracortical area (also described as a thymus dependent region—*Parrott & De Sousa* 1966) showed slight increase Similar increase was noted in the medullary cords, where also a number of large pyroninophilic cells were observed in few instances

Normal Recipients

Spleen and lymph nodes some increase in the number of secondary follicles in the

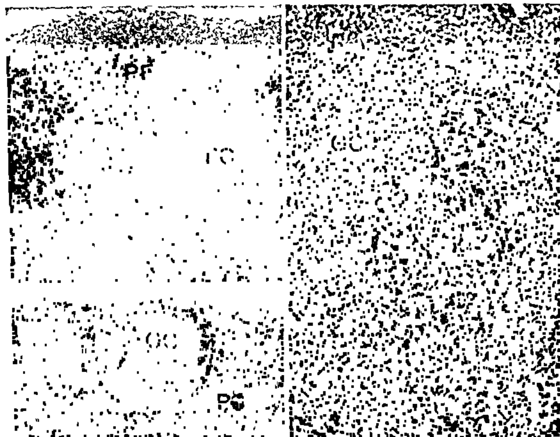


Fig 7 Section of lymph node of *nude* mouse, 6 days after heterotransplantation (rat skin) Primary follicles enlarged, confluent (PF), increased number of lymphocytes in paracortical area (PC) 80 ×, H&E

Fig 8 Section of lymph node from thymus grafted *nude* mouse, day 54 after thymus grafting Well developed germinal centres (GC) and lymphocytic infiltration of paracortical area (PC) 80 ×, H&E

Fig 9 Section of spleen, same animal as shown in Fig 8 Ample lymphocytic infiltration around arteriole (A) of Malpighian body Large germinal centre (GC) with signs of phagocytic activity (Arrows) 200 ×, H&E

spleen and lymph nodes, and accumulations of mononuclear cells in the medulla, were observed, but no other changes which could be described as characteristic

Thymus Grafted Nude *Mice Recipients*

Spleen in the periarteriolar (thymus-dependent) region large accumulations of mononuclear cells were observed, contrasting with the lymphocytic depletion in 'normal' *nude* mice Many germinal centres (secondary nodules) were also apparent with very active phagocytosis (Fig 9)

Lymph nodes reconstitution was apparently complete There were many secondary nodules, active phagocytosis, and massive lymphocyte infiltration of the paracortical region The medullary cords were swollen with mononuclear cells, and there were large numbers of large pyroninophilic cells especially around the sinuses of the paracortical region, and in the medullary cords (Fig 8)

Haematological Studies (Tables 1–4)

White blood cell counts are given for each of the recipients. There was always a leuco-

TABLE 6 *Cytotoxic Indices Day 30 of Sera from Rat Skin Grafted* Nude *and Normal Mice and Untreated* Nude *and Normal Mice*

Strain	No	*Nude*, skin grafted	Normal mouse skin grafted	*Nude* untreated	Normal mouse untreated
BALB/c	6723	0 88			
	6724	0 92			
	6727		0 98		
	HS12			0 00	
	HS02				0 20
C3H	6714	0 64			
	6715	0 70			
	6718		1 00		
	HS21			(0 10)	
	HS25				0 24
C57/BL	6705	0 88			
	6706	0 84			
	6709		1 00		
	HS42			0 00	
	HS33				(0 04)

Undiluted sera against Wistar rat thymus cells Values in brackets not significant

cytosis Both *nude* and normal mice displayed lymphocytosis from the 10th day onwards, but at day 6 the finding was not so uniform The tables give the actual individual counts

Cytotoxicity Tests

The results are given in Table 6 The Cytotoxicity Index (C I) is the usual parameter reflecting the number of specifically killed cells expressed in relation to the number of surviving cells in the complement control It appears from the tables that cytotoxicity of serum from the *nude* recipients was always marked of nearly the same order as that in serum from the normal recipients In only two of six not transplanted mice, both *nude* and normal was any spontaneous cytotoxic antibody identified and only in low titre

DISCUSSION

The findings are in accordance with those of earlier studies (*Rygaard* 1969 *Rygaard* & *Povlsen* 1969, *Povlsen* & *Rygaard* 1971, 1972 *Povlsen et al* 1973, *Giovanella et al* 1972 *Manning et al*, in print) in evidencing that *nu* gene homozygosity entails inability to reject heterotransplants, in this case grafts of normal skin from an inbred strain of rats

In an earlier study (*Rygaard* 1969) adult Wistar rat donors were used, and intermediate unpublished studies by the same author have shown that grafts from Wistar hooded and Fischer rats are all similarly and readily accepted irrespective of the age of donors There are, however, technical advantages in using neonatal donors because skin shaving is not then necessary Skin thickness is also similar to that in the *nude* mouse, and it is easier to appose donor and recipient skin edges

One reserve, which this study has shown to be unfounded was that antigenicity of grafts might not be fully developed Taking account of the cytotoxic antibody titres in both *nudes* and controls this would appear not to be the case

The summed results of the study are convincing Fortytwo of 42 grafts in not prepared *nude* mice were accepted (including second set grafts) The acceptance at this present

time seems to be independent of the genetic backgrounds of the *nude* mice

The normal recipients uniformly rejected the grafts, and this reaction was accelerated in the case of second set grafts

Of the 12 thymus grafted *nude* mice, 10 rejected the grafts—two rejections evaluated from microscopic study but not less certain for that—while two accepted the grafts Thymus grafting in these latter two failed No thymus tissue could be recorded and there were no signs of lymphoid reconstitution

The skin grafts in the not prepared *nude* mice induced changes in the lymphoid tissues, particularly evident in growth of the primary lymph node follicles Similar growth could not certainly be identified in the normal recipients where the transition to the paracortical zone is not clearly defined In the *nude* recipient again there was only modest lymphocyte migration to the paracortical region In the spleen, increased numbers of mononuclear cells were seen in small aggregations in the red pulp, but no changes were observed in the periarteriolar, thymus dependent area No attempt is made in this study to explain the monocytic infiltration of the red pulp There was some sparse development of secondary follicles in the untreated *nude* mice once evident on day 6, but otherwise delayed until day 10 or later

Two not prepared *nudes* displayed aggregations of large pyroninophilic cells in the medullary cords (*Scothorne* & *McGregor* 1955) Similar more marked aggregations were seen in five of the six recipients of second set grafts at day 60

The study also confirms that thymus grafts in *nude* mice confer capacity of rejecting rat heterografts This is as found in neonatally thymectomized mice, and in other *nude* mice (*Wortis* 1971, *Kindred* 1971) The thymus grafts also entail other consequences This study evidences that a reconstitution normalization of appearance of lymphoid tissue also occurs Secondary follicles appear often in larger numbers than observed in normal graft recipients the thymus dependent periarteriolar regions in the Malpighian bodies and in the paracortical zones of lymph nodes are repopulated by lymphocytes

A particularly interesting observation was the significant lymphocytosis which followed transplant in the not prepared *nude* mice Do these lymphocytes carry immunoglobulin of IgM type on their surfaces? Are they, in fact, B cells? To what extent, if any, will they function in cell killing or antibody production 'in vitro'?

A similar study involving allotransplantation (*Rygaard* 1974) shows that cytotoxic antibody production in response to allogeneic stimuli is very slight which contrasts with the finding herein reported of considerable cytotoxic antibody production in response to heterograft stimulus A comparison of the macroscopic and microscopic appearances of graft take and development in these two circumstances is striking Rat grafts appear to be more readily accepted than mouse grafts So far as regards the cytotoxic antibodies it is the question whether these can appear as enhancing antibodies (*Kaliss* 1958) 'in vivo' Experiments involving transfer of serum from heterotransplanted *nude* mice to normal recipients may possibly answer the question

That heterografts are more readily accepted than allografts is something of a paradox *Lafferty* & *Jones* (1969) recorded a finding which may be related In experiments involving simultaneous transplantation of embryonic chicken and pigeon tissue onto the chorio allantoic membrane of fertilized chicken eggs, the addition of allogeneic lymphocytes led to rejection of the allogeneic transplant while the heterogeneic survived

These two studies and other, suggest that biological alloaggression is more powerful than heteroaggression that histocompatibility and immune recognition systems are complementary as proposed by *Jerne* (1971)

The expert assistance of Miss *Inger Bull* and Miss *Margit Bæksted* and Mr *Oluf Rasmussen* s constant care for the animals is gratefully acknowledged Dr *Eva Klein* and Miss *Susanne Becker* gave their valuable advice and assistance during my stay in

the Department of Tumour Biology, Karolinska Institutet, Stockholm

This study was supported by *The Danish Cancer Society* and the *Danish Medical Research Council*

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SKIN GRAFTS IN *NUDE* MICE

3 Fate of Grafts from Man and Donors of Other Taxonomic Classes

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Avian, reptilian and normal human skin grafts are well accepted by the *nude* mouse, as demonstrated by this and other studies Histological studies show consistently good incorporation of grafts, and changes in spleen and lymph nodes similar to those observed after other transplants in the animal The consensus of studies evidence that the *nude* mouse is the least resistant recipient of grafts of diverse origin among the experimental animals, prepared or otherwise, which are under study today This is an expression of the extent of the immunological deficiency in the *nude* An interesting question asked by *Calne* in 1970 can be answered Though its 'milieu interieur' could be thought to be widely different from the avian and the reptilian, the *nude* mouse is an adequate host of avian and reptilian tissue

Allogeneic and heterogeneic transplants in normal mice are shed after a classical process of rejection over a period of few days (*Loeb* 1918, 1945) The mouse mutant *nude* is born without a thymus, and will accept both allogeneic and heterogeneic transplants (*Rygaard* 1969, *Rygaard & Povlsen* 1969, *Kindred* 1971, *Wortis* 1971, *Pennycuik* 1971, *Pantelouris* 1971)

Previous studies have shown that *nu* gene homozygosity in mice, irrespective of the constitution of the remaining genetic spectrum, entails the incapacity to reject allografts, as evidenced by different donor recipient combinations (*Rygaard* 1974a), and incapacity to reject heterografts (rat skin), both first and second set grafts (*Rygaard* 1969, *Rygaard* 1974b) Other studies report the acceptance by *nude* mice of normal skin from the chicken (*Rygaard* 1971), and from the pigeon (*Rygaard* 1972), while it has also been shown that human malignant tumour grafts are accepted (*Povlsen & Rygaard* 1971, 1972, *Povlsen et al* 1973)

The following study describes the transplantation of normal skin from the chicken, the pigeon, the lizard, the grass snake and man to the *nude* mouse, and the outcome thereof

The grafts were regularly scrutinized macroscopically, and, at autopsy, spleen and lymph nodes were scrutinized, and preparations were made from graft, spleen and lymph nodes for histological study

Received 22 vii 73 Accepted 22 vii 73

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MATERIAL AND METHODS

Recipients

Nude mice These animals were seven to eight week old products of the fourth to fifth cycle in a transfer (*Snell* 1948, *Green & Doolittle* 1963) of the *nu* gene to the BALB/c/A/BOM$_f$ strain of inbred mice The animals were either bred at the Pathological Anatomical Institute, Kommunehospitalet, Copenhagen, Denmark, as previously scribed (*Rygaard & Friis* 1973), or from Gl. Bomholtgaard, DK 8680 Ry, F

Normal mice These were of similar age, of the inbred BALB/c strain, purchased from Gl Bomholtgaard

No account was taken of the sex of either *nude* or normal mice

The animals were housed under spf conditions but without personnel barriers They were kept separately in Makrolon®, type II cages with sterile wood granulate for bedding Food pellets, prepared and autoclaved at Gl Bomholtgaard, were provided, together with sterile drinking water *ad lib*

Details of husbandry have been recorded previously (*Rygaard & Friis 1973*)

Donors

Chickens Two to three day old chicks were used of the White Italian breed, or of White Italian/Sussex crosses. These were supplied by Veterinary Surgeon *K E Mollegård*, DK-4623 Ll Skensved, Denmark The two donor types are not differentiated

Pigeons Two day old, not inbred young were purchased from a commercial breeder

Lizards Representatives of the species *Lacerta gracilis* were purchased from a dealer

Grass snakes (*Natrix natrix*) were taken from their natural habitat

Human donor skin was obtained from Sct Joseph's Hospital and Surgical Department I, Kommunehospitalet, from patients undergoing surgery

Grafting Technique

Recipients were anaesthetized with ether or intraperitoneal Propanidid (Epontol®, Bayer AG BRD), 0 5 mg/g The animals were then laid on their stomachs on sterile drapes with no form of restraint A 12 × 12 mm area of skin was removed from the back, and the prepared grafts carefully cut to size, were placed so that skin edges apposed Initially grafts were held in place with 4/0 atraumatic sutures but latterly we have used the tissue adhesive Histoacryl® (Braun Melsungen, BRD) No bandages or muzzles were used

Preparation of Grafts

Chickens and pigeons were decapitated under deep ether anaesthesia The already apparent down was lightly trimmed after disinfection with 70 per cent ethanol Donor skin was cut to size and scraped clean of connective tissue and the majority of muscle with the sharp blade of a fine pair of straight scissors where after the graft was immediately laid in pl[illegible] and fixed

Lizards and s[illegible]es Hibernation was induced by keeping the rept[illegible] at + 4° C After decapitation skin was taken fr[illegible] the back lightly scraped, cut to size, placed an[illegible] fixed

Human donors Skin was taken from the thighs of two patients who had undergone high femoral amputation in treatment of arteriosclerotic gangrene of the leg The subcutis was scraped off, and the deeper aspect of the dermis removed with a scalpel The thus prepared skin was stored in physiological saline in a Petri dish at + 4° C until required

Observation

Animals were scrutinized daily and the appearance of grafts noted Naturally dying animals and those sacrificed (see tables), were autopsied Specimens of grafts, spleen and lymph nodes were taken for histological study Tissues were fixed in formalin, embedded in paraffin wax and 7 μm sections were stained with H & E and a m Van Gieson Hansen

Study Plan

Five series of studies were made of

1) 14 recipients of chicken skin Two recipients died naturally The remainder were sacrificed at the times noted in Table 1

2) 14 recipients of pigeon skin sacrificed as recorded in the table

3) 8 recipients of lizard skin One died naturally on day 30 The remainder were sacrificed as recorded in the table

4) 21 recipients of grass snake skin Four died naturally between days 18 and 21 The remainder were sacrificed as recorded in the table

5) 10 recipients of human skin Two died naturally on days 18 and 27 The remainder were sacrificed as recorded in the table

Controls in each of the five series four normal mice received similar transplants These animals were sacrificed at the same time as *nude* recipients or at the conclusion of studies

RESULTS

Macroscopic Observation, Nude Recipients

1) *Chicken grafts* At two days there was rich vascularization of grafts, and two days later healing in along the graft edge Feathers grew from the trimmed grafts to between one and three millimetres at about day 10 There was no further growth, in fact some loss, but feathers were preserved until the sacrifice day 75 From day 30 onwards moderate graft contraction was observed (Fig 1)

2) *Pigeon grafts* The course was much as described after chicken grafts Graft skin appeared thinner, with a mild degree of atrophy

TABLE 1 *Fate of Skin Grafts from Chicken, Pigeon, Lizard, Grass Snake and Man in Nude Mice of BALB/c Background*

Donor	Taxonomic class	No grafted	Natural death day	Accept	Sacrificed day	Accept	Total accept
Chicken	Aves	14	42, 55	2/2	4, 4, 8, 8, 8 *21, 21, 30,* 30, 59, 75, *107*	10/12	12/14
Pigeon	Aves	14	0		6, 6, 6, 6, 21, 21, 30, 30, 35 (6 mice)	14/14	14/14
Lizard	Reptilia	8	30	1/1	14, 16, 22, 25, 30, 30, 30	7/7	8/8
Grass snake	Reptilia	21	3, 18, 19, 27	4/4	8, 14, 14, 14, 21, 21, 30 (10 mice, *1 not accept*)	16/17	20/21
Human	Mammalia	10	18, 27	2/2	10, 28, 28, 30, 40, 40, 42, 42	8/8	10/10

Day of sacrifice in *Italics* = not accepted
Four normal mice grafted in each series all rejected the graft day 10–16

3) *Lizard grafts* There was early healing at the graft edge The pigmentation of the graft obscured the process of re-vascularization Between days 14 & 20 an event reminiscent of slough shedding was frequently observed A fine, nearly transparent, superficial layer of skin was shed At the same time a greyish rim appeared at the graft edge, probably reflecting an overgrowth of host epithelium The graft papillae were smoothed out, but still readily apparent Moderate graft contraction occurred after day 14

4) *Grass snake grafts* Similar course as with lizard grafts The skin shedding phenomenon was more marked, as was the epithelial overgrowth at the graft edge

5) *Human skin* These thick grafts first evidenced signs of revascularization between days six and 10—the appearance at the graft edges changed from a yellow pallor to normal colouration At the centre of all grafts a greyish-yellow necrotic scab formed Hair was preserved at the periphery, but lost centrally

Control animals In all the donor/recipient combinations, signs of rejection were manifest Grafts became necrotic, were lifted above the graft beds by marked oedematous swelling and proliferation, and subsequently shed

Autopsy

Irrespective of donor type, moderate splenomegaly and enlargement of, particularly regional, lymph nodes was apparent No specific pathological changes, which could be related to the transplantation, were observed

Histology

1) *Chicken grafts* On day 4 there was moderate oedema, and marked proliferation of fibrous connective tissue in the graft bed The connective tissue was infiltrated by granulocytes and histiocytes, but only few mononuclear cells were present On day 8 the proliferative and infiltrative processes were

ıg 1



ıg 3





Fıg 1 *Nude* mouse carryıng chıcken skın graft, day 21

Fıg 2 Sectıon of chıcken skın graft ın *nude* mouse, day 21 Well preserved feather follıcle (F) Few mononuclear cells ın stroma 125 ×, H&E

Fıg 3 Chıcken skın graft ın *nude* mouse, day 75 Atrophıc feather follıcle cut tangentıally (arrow) Well developed erector muscle (E) 80 ×, H&E

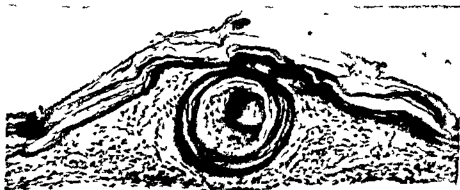


Fıg 4 Pıgeon skın graft ın *nude* mouse day 30 Atrophıc feather follıcle wıth low stratıfied epıthelıum wıth cornıficatıon 80 ×, H&E



Fig 5 Lizard skin in *nude* mouse day 30 Light flattening of papillae Normally appearing epithelium Melanotic pigment *in situ* No cellular infiltration of dermal tissue 200 ×, H&E

resolving Feather follicles (Calamus) were well preserved and there were no inflammatory cells to be seen (Fig 2) Musculature (the erector muscles) was well preserved From days 21 to 30 progressive atrophy of feather follicles was apparent, but the adjacent connective tissue and erector musculature was vital and normal and the epithelium was well developed No further changes were observed up to days 59 and 75 (the longest observed animals—Fig 3) Some few *feather follicles* were preserved in this stable state

2) *Pigeon grafts* Appearances were largely as in chicken grafts, but *feather follicle* atrophy was more marked—there were large oval cyst formations with a cornifying flat one—two layer epithelium Occasionally, there were keratin plugs in the lumina (Fig 4)

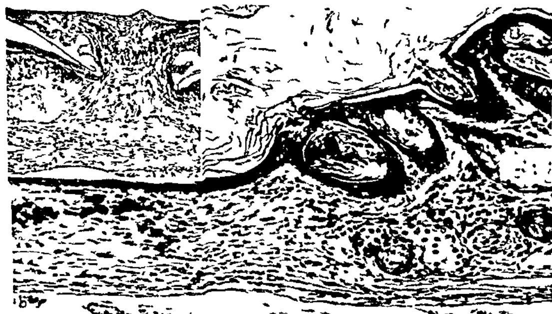
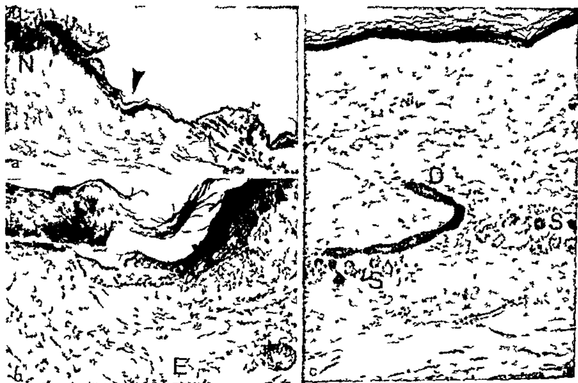


Fig 6 Grass snake skin in *nude* mice a Day 8 Graft incorporated in graft bed Papilla preserved but with incontinence of pigment announcing degeneration 100 × H&E b) Day 30 Recipient skin (right) fusing with flattened graft Connective tissue of graft dermis intact No papillae preserved 250 × H&E



F g 7 Human sk n n *nude* mice a) Day 10 Graft healed in completely Rec p ent sk n (right) and ad a cent part of graft appear normal Central part of graft necrot c (N) 12 × H&E b) Deta l of (a) a arrow The ep thel a are fus ng Moderate oedema of dermal tissue Well preserved erector musc e (E) no cellular react on 80 × H&E c) Day 42 Moderate flatten ng of graft Ep thel um and de m s appear normal—not infiltrated S veat glands (S) and duct (D) of normal s ructu e 80 × H&E

3) *Li ard grafts* The earliest studies were made on days 14 to 16 at which time there was full incorporation of the grafts with only minimal fibrous t ssue proliferation ai d granulocyte and hist ocyte infiltration Skin pap l lae were well preserved The epithelium was vital Tl ere were melanophores high lying in the dermis with grey brown or black pigment which showed no s gns of incontinence Dermal connective tissue was well preserved Capillaries of i ormal appearance med ated a rich blood supply Later (days 22 to 30) tl ere was some flatten ng of papillae but tl e pigment rema ned in situ and tl e derm s was vital At the margins tl ere was broad contact between host and donor epitl elium The origin of the graft epidermis could not be determined (F g 5)

4) *Grass ake grafts* Similar course to that descr b l n lizard grafts but the most superficial l orizontal aspects of tl e skin pa p llae showed a tendency to atropl y and tl e subsequent smooth ng out of pap llae was more marked Tl e derm s was well preserved and no cellular react ons were apparent (Fig 6)

5) *Human skin* Superf cial necros s at the centre of grafts was a constant f nd ng as was preservat on of marg nal and basal v tal ty with no s gns of cellular react on Vi al ty was evidenced by the appearance of tl e dermal connective t ssue and of the s e t glands Of these latter tl ose ly ng deep ere of normal appearance at the time of the latest studies (days 40 to 42) as were the deeper ly ng ducts The superf c al aspects of tl e ducts were however atropl c Tl e appearance of tl e erector muscles also reflected tl e vital ty of the grafts Tl e border between vital and necrot c graft t ssue was marked by

a belt of granulocytes and histiocytes Apart from this, there was no other cellular infiltration in the dermis (Fig 7)

Control animals all histological studies showed the classical picture of graft rejection

Spleen and Lymph Nodes

Nude recipients The appearances were as described previously (*Rygaard* 1974 a, b) Primary follicles in the lymph nodes were enlarged, and formed a broad confluent subcapsular rim Increased numbers of lymphocytes were seen in the paracortex of lymph nodes, and in the red pulp of the spleen Some lymphocyte aggregations appeared in the medullary cords of the lymph nodes and in some instances, formation of germinal centres (secondary nodules) were observed in both lymph nodes and spleen

DISCUSSION

The study confirms previous reports (*Rygaard* 1971, 1972) of the inability of the thymusless *nude* mouse to reject avian transplants (chicken and pigeon) It is further shown that reptilian transplants—from lizard and grass snake—are similarly accepted Finally, it is found that the acceptance of human tissue is not confined to human malignant tumours (*Rygaard* & *Povlsen* 1969, *Povlsen* & *Rygaard* 1971, 1972, *Povlsen et al* 1973), but that normal human skin is accepted with equal facility These findings agree in general with those of *Reed* & *Manning* (in print) who demonstrate that normal human skin is hosted by *nude* mice for the extent of their normal life span *Manning et al* (in print) report further the successful transplantation of skin from the cat, the chicken, the chameleon, the lizard, the tree frog (amphibia)

This success with the tree frog makes a contrast of some interest with the findings of this author (*Rygaard* unpublished) in studies of a frog—*Bufo viridis* In a series of experiments, the *nude* mouse consistently failed to accept the frog skin as clearly evidenced histologically by a marked belt of separation between graft and graft bed, composed of large numbers of granulocytes Despite this finding, at 10 days the graft evidenced every sign of vitality Dermis and epidermis were of normal appearance Grafts 'conventionally' rejected display total structure loss at this time It may be that there is no basic lack of agreement in the findings of the two studies *Manning et al* used the tree frog The skin of *Bufo viridis* does not usually tolerate drying, and it may be that a simple difference in physical state explains the apparent discrepancy The study herein reported, and those referenced, evidence that the capacity of the *nude* mouse to accept transplants is qualitatively and variously greater than that of the neonatally thymectomized mouse (*Miller* & *Dukor* 1964), or the thymectomized animal treated with antilymphocyte serum (*Lance* & *Medawar* 1968)

In partial summary, study of this mouse mutant *nude* evidences

1) that the animal's 'spontaneous' immunological incompetence is more extensive than can be induced in other animals by neonatal thymectomy or by immunosuppressive treatment, and

2) that the difference between the 'milieu interieur' of *nude* mice and the donors derived from the three taxonomic classes discussed herein is not such that transplantation fails

Calne (1970), discussing the possibility of transplantation between widely disparate species questioned whether ' the milieu interieur of the recipient would provide the graft with a satisfactory environment and all the necessary metabolites for its normal function, if one assumed that the discordant destructive reaction could be prevented '

This question can now be answered in the affirmative

I wish to thank Miss *Inger Bull* and Mr *Oluf Rasmussen* for expert assistance Messrs *Henrik Manley* and *Kåre Rygaard* were always as pleased to be relieved of their captive grass snakes as I was to receive them'

The study was supported by *The Danish Cancer Society* and *The Danish Medical Research Council*

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Acta path microbiol scand Section A 82, 113–115, 1974

ADENOACANTHOMA OF THE SMALL BOWEL

Report of a Case

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A case of adenoacanthoma of the small bowel is reported In light of a macroscopic and histological examination, the possibility of a primary tumour is discussed The conclusion is that the tumour originated in the small intestine

Adenoacanthoma is a carcinoma, in which both glandular and squamous elements are found In the gastrointestinal tract adenoacanthomas are rare They are found especially in areas, where squamous and glandular epithelium are contiguous as at the cardioesophageal junction (*Russi* & *Corcoran* 1950) and in the anal canal (*Morson* & *Volkstädt* 1963) Furthermore they have been described in other parts of the esophagus (*Kay* 1968), in the stomach (*Boswell* & *Helwig* 1965), (*Sōgi* 1959) and colon (*Cook* 1958) To my knowledge however adenoacanthomas have never been described in the small intestine

CASE REPORT

80 year old woman previously well For three weeks she had suffered from abdominal pains and three days before admittance she developed vomiting and constipation X ray examination disclosed ileus with numerous fluid levels in the small intestine The patient was operated as an emergency cases At laparotomy a tumour measuring about 10 cm in diameter was revealed It was localized to the middle part of the small intestine Proximal to the tumour the intestine was distended distally it was collapsed A heavy venous congestion was seen in the whole intestine, but unmistakable peristalses were present The tumour was adherent to the back wall of the abdominal cavity and was considered inoperable Two palliative anastomoses were performed The operation was uncomplicated, but fifteen days later the patient suddenly collapsed and died

Autopsy showed that the cause of death was pulmonary embolism

In the abdominal cavity pronounced adherences were found An ill defined tumour measuring about 10 cm in diameter was revealed approximately in the middle part of the small intestine The tumour was yellow and very necrotic with only a few solid areas Several loops of the small bowel passed into the tumour and other loops were adherent to the surface Macroscopically the tumour seemed to originate from the small bowel The tumour was adherent to the back wall of the abdominal cavity It extended into the mesenterium but no enlarged lymphnodes were seen In spite of careful examination no further tumours were found

Microscopic examination revealed a carcinoma consisting of two different elements (Fig 1) In some areas especially close to the surface of the intestine adenocarcinoma was seen This part of the tumour was made up of atypical glandular cells (Fig 2) Many of the glands contained mucus which was stainable with Alcian blue and PAS reaction In other parts of the tumour a pure squamous cell carcinoma was found A pronounced keratinization with horn pearl formations was seen in some areas (Fig 3) but in others the squamous cells were undifferentiated with big nearly bizarre nuclei In both components of the tumour a large

Received 26 xi 73 Accepted 18 ix 73

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Fig 1 Two different elements are seen in the same area of the tumour In the upper part of the figure a squamous cell carcinoma is seen, in the lower part adenocarcinoma (H and E × 100)

Fig 2 Adenocarcinoma made up of atypical glandular elements lined by one or several layers of cylindrical cells (H and E × 100)

Fig 3 Squamous cell carcinoma. Pronounced keratinization and horn pearl formations are seen (H and E × 100)

Fig 4 Transition between necrotic but non neoplastic intestinal mucosa and the carcinoma in this place made up of an adenocarcinoma (H and E × 40)

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number of atypical mitoses were seen The carcinoma penetrated all layers of the bowel wall In some sections it was possible to see transition between atypical necrotic but non neoplastic intestinal mucosa and the tumour tissue suggesting that the carcinoma originated from the small bowel

DISCUSSION

The microscopic diagnosis of the tumour was adenoacanthoma of the small bowel, but the unusual location of the tumour makes it important to rule out the possibility of a metastasis Adenoacanthomas are mainly met within the female genital tract, but in this case a careful macroscopic and histological examination failed to reveal any tumour here or at any other places Furthermore the microscopic examination suggested that the tumour originated from the intestine The fact that only one tumour was found seems to point toward a primary lesion, although it does not preclude a metastasis *De Castro et al* (1959) found the ratio between multiple and single metastases to the small bowel to be 12/14

As for the histogenesis of adenoacanthomas in the small bowel several possibilities must be considered The tumour tissue could develop in heterotopic gastric mucosa or pancreatic rests in Meckel's diverticulum It could originate in ectopic pancreatic tissue in other places of the small bowel, in areas of intestinal endometriosis or in relation to squamous metaplasia The microscopic examination of the tumour did not support any of these possibilities. Direct neoplastic stimulation of undifferentiated basal cells in the intestinal mucosa (*Wood 1943*) seems to be the most probable explanation

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I should like to thank dr *Aage Johansen* for his help and interest

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Acta path microbiol scand Section A 82, 116–126, 1974

THE IMMUNE REACTION AGAINST MALIGNANT MELANOMA STUDIED IN A BIOPSY MATERIAL

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Biopsies from primary and secondary malignant melanomas have been studied by light microscopy as well as by fluorescence and electron microscopy In the two primary tumours, lymphocytes in different stages of transformation were observed at the periphery of the tumour, but evidence of a cytotoxic effect of the immune cells against the tumour cells could not be obtained Plasma cells were few and deposits of γ globulins were not observed outside these cells In 13 of the 15 metastatic tumours, lymphocytes and plasma cells were observed among the tumour cells, the latter being the most prevalent cell type In 11 patients, γ globulins were found on the surface of the tumour cells and in 7, a variable proportion of the cells showed specific cytoplasmic fluorescence In one tumour, ultrastructural changes, interpreted as changes due to immune cytolysis, were observed Viruslike particles were not observed in any case

In vitro studies have demonstrated both humoral (*Morton et al* 1968, *Lewis et al* 1969, *Romsdal & Cox* 1970) and cellular immunity (*Hellstrom et al* 1968, 1971 a, *Fass et al* 1970, *Fossati et al* 1971, *de Vries* 1972) against malignant melanomas The antibodies react with both the cellular membrane, the cytoplasm and the nucleus of the tumour cells These reactions are observed *in vitro*, but one would also expect them to take place *in vivo* i e in the tumour tissue The aim of the present study was to examine this possibility in a biopsy material using morphological methods

Received 1 vii 73 Accepted 15 viii 73

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MATERIAL AND METHODS

A total of 17 cases of malignant melanoma was studied Two of these were primary tumours and 15 were metastatic tumours

At the time of removal one piece of the biopsy was cut in approximately ½ mm^3 cubes and fixed in 3 per cent glutaraldehyde in 0 2 M cacodylate buffer pH 7 4 Another piece was trimmed into 1 mm^3 cubes and transferred to phosphate buffered saline (PBS) pH 7 2 Lastly, a larger piece was fixed in 4 per cent formaline

The formaline fixed tissue was sectioned and stained for light microscopic study

The glutaraldehyde immersed material was fixed for 2–3 hours Then it was transferred to cacodylate buffer alone overnight, and the next day post fixed for 1 hour in 1 per cent OsO_4 in S-collidin buffer The blocks were dehydrated, embedded sectioned and stained for electron microscopy as previously described (*Kåresen* 1970) The blocks immersed in PBS were kept at 4° C for 48 hours during which PBS was changed twice This was done to remove the diffusible serum components

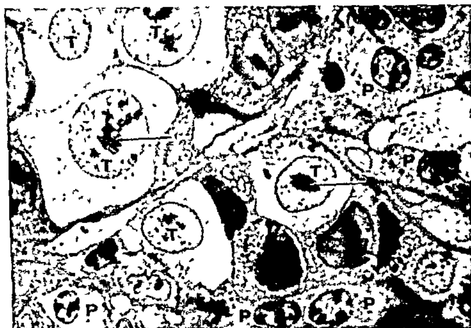


Fig 1 Light microscopic picture of a metastatic melanoma The tumour cells (T) with abundant cytoplasm and prominent nucleolus (arrows) are easily identified In between the tumour cells, plasma cells (P) with the typical cog wheel appearance of the nuclear chromatin are found × 660

from the tissue (*Brandtzaeg et al* 1966) The blocks were then fixed in 95 per cent alcohol, embedded in paraffin, sectioned and prepared for fluorescence microscopy as described by *Sainte Marie* (1962) The sections were incubated for ½ hour with fluorescein isothiocyanate labelled rabbit anti human γ globulin serum The conjugate had F/P ratio 2 7 and a haemagglutinating titre 1/3200 against γ globulin coated red cells It showed a single line against human full serum in immunoelectrophoresis After thorough washing the sections were studied and photographed with a Leitz Orthoplan fluorescence microscope fitted with a 1 5 mm BG 12 exciter filter, a 495 μm interference filter and a K 510 barrier filter

RESULTS

The diagnosis malignant melanoma was confirmed in all biopsies by light microscopy In most sections plasma cells were found surrounding the tumour and in its connective tissue In 5 of the metastatic tumours plasma cells were rather abundant (Fig 1) Lymphocytes were, however, more rarely seen On the contrary, the two primary melanomas had a rather heavy infiltration of lymphoid cells at the periphery of the tumour

Fluorescence Microscopy

In accordance with the light microscopic results, small cells with strong green cytoplasmic fluorescence, most probably plasma cells, were seen in and around 13 of the 15 secondary tumours (Fig 2) In the primary melanoma, only a few fluorescent cells were observed in the peripheral leucocyte infiltrate

In 11 of the 15 metastatic tumours, specific green fluorescence was observed on the surface of the tumour cells, mainly as small spots (Fig 3), but occasionally as a continuous ring around the periphery of the cell (Fig 2 and 3) This was not found in the primary melanoma

In 7 of the 15 metastatic tumours, the tumour cells exhibited a strong green cytoplasmic fluorescence (Fig 2 and 3) The number of fluorescent cells varied from tumour to tumour, from less than $^1/_{10}$ to

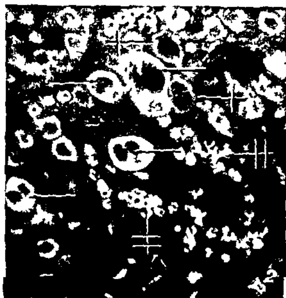


Fig 2 Section from a metastatic melanoma incubated with fluorescein conjugated anti human γ globulin serum Tumour cells with large non fluorescent nuclei and strongly fluorescent cytoplasm can be found (arrows) Groups of smaller cells appearantly plasma cells are also seen (double barred arrows) Not all of the tumour cells are fluorescent (arrow heads), however, and some only have a thin fluorescent ring at the cell periphery (cross barred arrows) × 310

more than ⅔ tumour cells Cytoplasmic fluorescence was not seen in the primary melanoma

Specific fluorescence in the nuclei was rare and if observed, it would be localized exclusively in or near necrotic areas of the tumour (Fig 3) In the necrotic areas amorphous masses of green fluorescent material were also regularly found (Fig 3)

Electron Microscopy

Electron microscopy revealed three different cell categories both in primary and secondary melanomas 1 Tumour cells with a general appearan e similar to that described by others (*Cesari* ı 1971 *Klug* & *Gunther* 1972) (Fig 4 and 5)

2 Cells with a ı ore electrodence cytoplasm and rather abu nt endoplasmic reticulum They containeu variable amount of pigment in vesicles which were probably of endocytic origin as they varied considerably in size and in amount of pigment It is assumed that these cells belonged to the connective tissue of the tumour (Fig 4 and 5)

3 Leucocytes In the primary malignant melanoma, these cells were found only in the periphery of the tumour, and they consisted mainly of lymphocytes in different stages of transformation (Fig 6) Most of them had rather abundant cytoplasm but sparse endoplasmic reticulum and few polyribosomes (Fig 6) i e in the early stages of transformation Others, however, had a more well developed endoplasmic reticulum and numerous polyribosomes i e approaching the appearance of immunoblasts (*Moıat* & *Fernando* 1965, *de Petris et al* 1966) Cells in different stages of the development towards plasma cells were also seen but more rarely (Fig 6) Scattered macrophages could also be found, often containing large amounts of pigment (Fig 6) In the secondary malignant

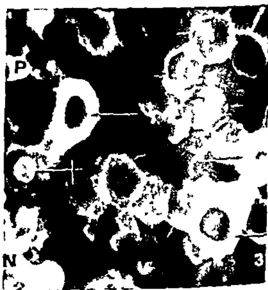


Fig 3 Material and treatment as Fig 2 Three tumour cells with strongly fluorescent cytoplasm can be observed (arrows) Others only have a thin ring or small spots fluorescing at the cell periphery (arrow heads) Close to a necrotic portion of the tumour (N) a single cell showing nuclear fluorescence may be found (cross barred arrow) A plasma cell can also be seen (p) × 390

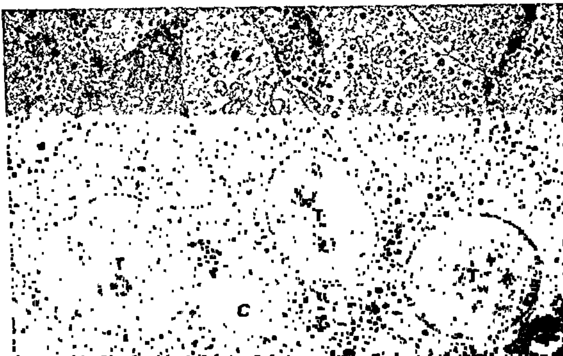


Fig 4 Electron micrograph of a primary malignant melanoma The tumour cells (T) have a fairly electron translucent cytoplasm and contains a few melanosomas (arrows) A connective tissue cell can also be seen (C) × 3700

melanoma, the leucocyte infiltrate was dominated by well developed plasma cells (Fig 7) However, lymphocytes in different stages of transformation were also found (Fig 7, 8 and 10). Contrary to the observation in the primary melanoma, the leucocytes were also seen in between and often in close contact with the tumour cells (Fig 8, 9 and 10)

In one of the metastatic tumours, the one showing the most extensive cytoplasmic fluorescence, a process that appeared to be of cytolytic nature was observed in the tumour cells Different stages in this process were observed In some of the tumour cells, the change was restricted to a small area of the cytoplasm which appeared looser than the rest (Fig 8, 9 and 10) In others, most of the cytoplasm had this loose appearance, often containing large vesicles with myelin figures and other cell remnants, presumably of autophagic origin (Fig 10) Lastly, tumour cells could be observed where the whole cytoplasm was filled by such vesicles and with partly disrupted cell membranes (Fig 7).

These features were observed in tumour cells which were surrounded by normal appearing cells, and in sections where no sign of necrosis in surrounding areas could be found

Often (Fig 8 and 10), but not always (Fig 10), cells identified as lymphocytes were situated in close contact with the cytolytic tumour cells If only part of the tumour cells seemed damaged, the affected area was mainly found directly beneath the lymphocyte (Fig 8, 9 and 10)

Viruslike particles were searched for in the tumour tissue, but were not observed in any case.

DISCUSSION

In vitro studies of human reactions against malignant melanomas have demonstrated the presence of both the cellular (*Hellström et al.*



Fig 5 Larger magnification of the central area in Fig 4 showing the sparse melanosomas (arrows), the prominent nucleolus (N) and the sparse strands of endoplasmic reticulum (arrowheads) in the tumour cell (T) The connective tissue cell (C) has a more abundant endoplasmic reticulum (arrow heads) and the pigment containing vesicles (arrows) vary more both in size and in electron density than in the tumour cell × 12000

1968, 1971 a, *Fass et al* 1970, *Fossati et al* 1971, *de Vries* 1972) and the humoral (*Morton et al* 1968, *Lewis et al* 1969, *Romsdal* & *Cox* 1970) type of reaction The circulating antibodies are directed towards the cellular membrane, the cytoplasm and the nucleus Such antibodies may be cytotoxic to the tumour cells, but may also protect them from the cytotoxic effect of the immune lymphocytes i e blocking antibodies (*Hellstrom et al.* 1971 b).

However, as these immune products must be present in vivo one would expect to find accumulations of both lymphocytes and γ-globulins in tumour biopsies This can in fact be demonstrated

In primary, malignant melanomas, an infiltration of lymphoid cells in the periphery of the tumour is usually observed (*Pajan et al* 1970) This was also found in the two primary tumours of the present material The electron microscopic study demonstrated that the lymphocytes were in different stages of transformation from the small inactive lymphocyte to the immunoblast Such transformation of the lymphocytes is found where an immune reaction takes place (*Hall et al* 1967, *Turk* 1967).

However, with the exception of a few plasma cells, accumulation of γ-globulins was not observed in the primary tumours This could have been due to the fact that the concentration of γ-globulin was too low to be detected by the immunofluorescence technique Alternatively the patients have not had circulating antibodies Neither could we find



Fig 6 Survey picture of the leucocyte infiltrate at the periphery of the primary malignant melanoma Most of the cells are lymphocytes (L) in the early stages of transformation with an abundant cytoplasm, but with sparse endoplasmic reticulum and few mitochondria Immunoblasts (I) with numerous polyribosomes, macrophages (M) with vesicles containing a variable amount of phagocytosed pigment, and occasional cells from the plasma cell series, here pro-plasmablasts (P) are also found × 5100

evidence of a cytotoxic reaction of the lymphocytes against the primary melanoma cells This could be due to sampling difficulties As the lymphocytes were located at the periphery of the tumour, and as lymphocyte mediated cytotoxity is based on a direct lymphocyte target cell contact (*Shacks & Granger* 1971), immune cytolysis could be expected only on the borderline between tumour and lymphocyte infiltrate The number of blocks obtained from this area was restricted, and thereby the number of examined

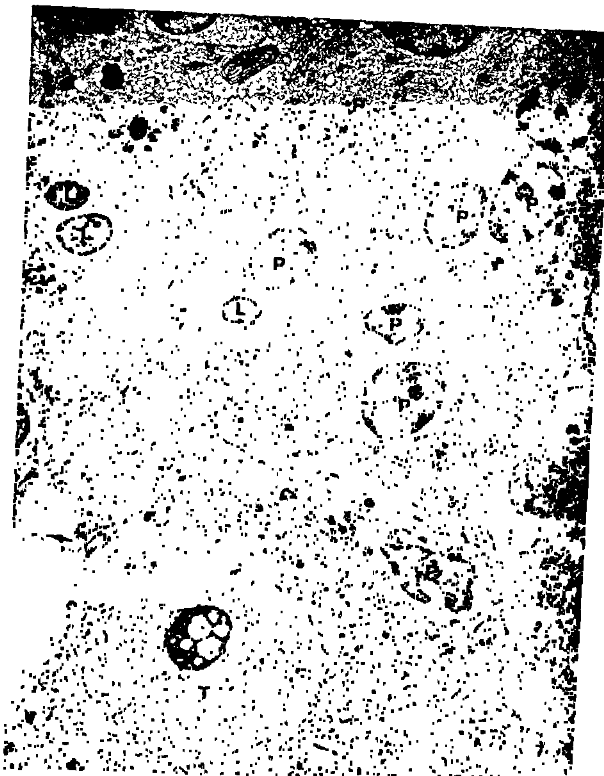


Fig 7 Section from a metastatic malignant melanoma showing the preponderance of plasma cells (P) among the leucocyte infiltrate A few lymphocytes (L) are, however also found The tumour cell (T) exhibits an extensive cytoplasmic vacuolization, and its cell membranes are partly disrupted × 4400

Fig 8 Section from a metastatic malignant melanoma showing lymphocytes (L), immunoblast (I), and plasma cells (P) in close contact with the tumour cells (T) Arrows point to areas of the tumour cell cytoplasm which appear looser than the rest × 3900

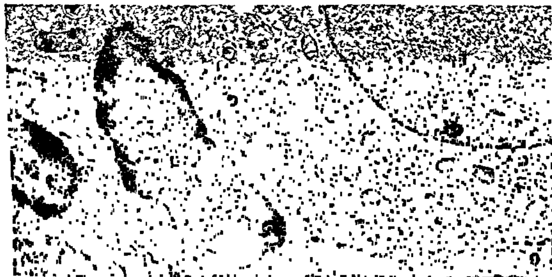


Fig 9 Larger magnification of the central area in Fig 8 showing the close contact between the lymphocyte (L) and the tumour cell (T) The looser texture of the tumour cell cytoplasm directly beneath the lymphocyte is appearant × 11400

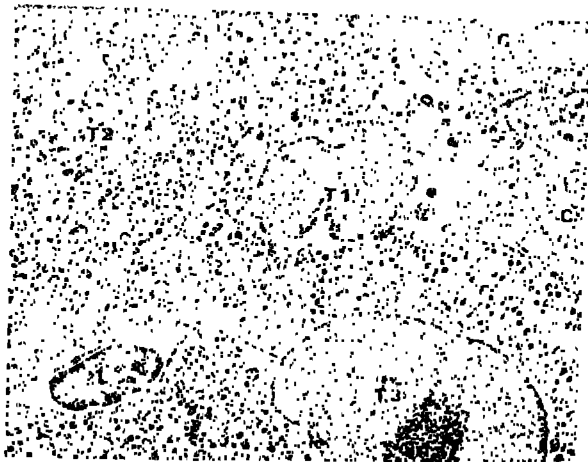


Fig 10 Section from a metastatic melanoma exhibiting a tumour cell (T 1) where most of the cytoplasm is affected by a cytolytic process Vesicles of variable size containing cell debris can be seen Another tumour cell (T 3) is in contact with a lymphocyte (L) and its cytoplasm is less electron dense than the rest in a small area close to the immune cell (arrow) Surrounding tumour (T 2) and connective tissue cells (C) have a normal appearance × 4200

tumour cells in contact with lymphoid cells Also in the metastatic tumour, findings indicated an immune reaction in the form of infiltration of lymphoid and plasma cells among the tumour cells This infiltration was also observed in subcutaneous metastases, and simple trapping of lymphoid cells by the tumour during invasion of a lymph node can thus be excluded It is interesting to note that the ratio of plasma cells to lymphoid cells seemed to be much higher in the secondary than in the primary tumours Whether this observation has any connection with the "blocking antibodies" found in the serum of patients with disseminated melanomas (*Hellstrom et al* 1971 b) is not known It may, however, be speculated that this shift from lymphoid to plasma cell dominance could be the morphological correlate to the appearance of such "blocking antibodies" in the disseminated tumours The results of the studies by the immunofluorescent technique may give some support to this speculation γ-globulins were found on the surface of the tumour cells in 11 of 15 metastatic melanomas As the electron microscopic study did not reveal cytolytic phenomena in these tumours, the surface bound γ-globulins might have been of the blocking type.

On the other hand, green cytoplasmic fluorescence was found in 7 of the 15 secondary deposits showing the presence of γ-globulins

in the cells As γ-globulins are not able to penetrate an intact cell membrane into the cytoplasm (*Goldberg* 1963), one must assume that the γ-globulin containing cells were damaged, perhaps through the immune reaction Ultrastructural changes indicating a cytolytic process, however, were found in only one of these tumours As the number of tumour cells showing cytoplasmic fluorescence was rather variable in the different tumours, from less than $^{1}/_{10}$ to more than $^{2}/_{3}$ the total, this lack of detectable cytolysis could be due to sampling difficulties, as suggested for the primary melanoma In fact, the only tumour where a cytolytic process with certainty could be found, was identical with the one showing the most extensive cytoplasmic fluorescence

Our observations of γ globulins in association with the tumour cells are in contradiction to the results obtained by *Kopf et al* (1966) Using the fluorescent antibody technique on primary and secondary malignant melanomas, they found γ globulins only in plasma cells and large mononuclear cells

The possibility that the cytolytic changes observed could be due to anoxia cannot be excluded However, the cytolytic tumour cells were repeatedly found to be completely surrounded by normal appearing cells well away from more extensively necrotic areas Combined with the observation that cells identified as lymphocytes regularly were found in direct contact with the damaged cells, this gives, according to our opinion, support to the idea that the cytolysis was due to an immune reaction and not to anoxia

Viruslike particles have been observed in transplantable melanomas of experimental animals (*Epstein & Fukuyama* 1970), and virus have been suggested as an aetiologic factor in human malignant tumours (*Allen & Cole* 1972) We therefore searched rather thoroughly for such particles in the present material, but without success

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The biopsy material was supplied by Dr *I Brennhovd* and Dr *O Jorgensen* and the fluorescein conjugated anti γ globulin serum by Dr *H Ørjaseter* I owe them my best thanks The technical assistance of Miss *Karin Skaug* is also appreciated

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Acta path microb ol scand Section A 82, 127–136, 1974

CARCINOGENICITY IN MICE OF SOME FATTY ACID METHYL ESTERS

I Skin Application

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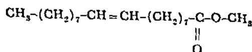
Two oxygen-containing derivatives of unsaturated fatty acids methyl 12-oxo *trans* 10 octadecenoate and methyl hydroxyoctadecadienoate, both highly active in the newt test for carcinogenicity, have been tested as complete carcinogens and as promoting agents by application to the skin of ST/a mice Through observation for two years the tumourigenic effect was recorded and compared with that of methyl oleate and of adequate positive control substances 7,12 dimethylbenz[α]anthracene and croton oil Both methyl oxo-octadecenoate and hydroxyoctadecadienoate showed a significant potency to promote the induction of skin papillomas The genesis of malignant skin tumours was promoted by methyl oleate and oxooctadecenoate and all the three methyl esters had a weak activity as complete carcinogens to the skin As a systemic effect all the esters promoted the induction of malignant lymphomas most markedly methyl oxooctadecenoate Even without preceding initiation this ester and methyl oleate had some activity in lymphoma cancerogenesis The various observations show that when tested by our experimental procedures methyl oxo octadecenoate is the ester most active in the induction of tumours and the effect is essentially that of a promoter The relation between molecular configuration and carcinogenicity of fatty acids is discussed

The newt test has been used as a method for screening lipids in order to sort out substances suspect of carcinogenic properties A number of altered lipids derived from unsaturated fatty acids by autoxidation and related processes was tested (*Glavind* & *Arffmann* 1970) The most active compound was methyl 12-oxo *trans* 10 octadecenoate which can be derived from methyl oleate by isomerization and the introduction of an oxo group A second compound highly active in the newt test was methyl hydroxyoctadecadienoate which can be derived from methyl linoleate by isomerization and the introduction of a hydroxyl group (Figure 1) It can be prepared from methyl linoleate hydroperoxide by reduction, whereby the hydroperoxide group is converted into a hydroxyl group Methyl linoleate hydroperoxide showed an activity in newts similar to that of the hydroxy compound, but was more toxic (*Arffmann* & *Glavind* 1970)

Definite establishment of carcinogenicity requires long term bioassays in mammals, preferably using various routes of administration (*Berenblum* 1969) Testing of methyl 12 oxo-*trans* 10-octadecenoate and methyl hydroxyoctadecadienoate together with methyl oleate as a supposedly non carcinogenic control substance, is now in progress, and

Received 20 xi 73 Accepted 24 xi 73

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Methyl 9-octadecenoate (Methyl oleate)

$CH_3-(CH_2)_5-\underset{\underset{O}{\|}}{C}-CH=CH-(CH_2)_8-\underset{\underset{O}{\|}}{C}-O-CH_3$

Methyl 12-oxo-*trans*-10 octadecenoate

$CH_3-(CH_2)_4-CH=CH-CH_2-CH=CH-(CH_2)_7-\underset{\underset{O}{\|}}{C}-O-CH_3$

Methyl octadecadienoate (Methyl linoleate)

$CH_3-(CH_2)_4-\underset{\underset{O\ OH}{|}}{CH}-CH=CH-CH=CH-(CH_2)_7-\underset{\underset{O}{\|}}{C}-O-CH_3$

Methyl hydroperoxidooctadecadienoate (Methyl linoleate hydroperoxide)*

$CH_3-(CH_2)_4-\underset{\underset{OH}{|}}{CH}-CH=CH-CH=CH-(CH_2)_7-\underset{\underset{O}{\|}}{C}-O-CH_3$

Methyl hydroxyoctadecadienoate*

* The methyl linoleate hydroperoxide prepared by autoxidation and the methyl hydroxyoctadecadienoate prepared by reduction of the hydroperoxide are mixtures of isomeric compounds, substituted at the 13 position, as in the structures presented above, or in the 9 , 10 , 11- or 12 positions

Fig 1 Chemical structures of fatty acid methyl esters

some preliminary results have been published (*Arffmann & Glavind* 1971) Mouse skin was chosen as a target organ in the first long-term experiments The application to mouse skin has the advantage of being a testing method systematized to distinguish between complete carcinogens and promoting agents (*Berenblum* 1969)

MATERIALS AND METHODS

Substances The preparation of methyl 12 oxo-*trans* 10 octadecenoate was carried out by a modification of the method of *Nichols & Schipper* (1958)

12 Oxo-*cis* 9 octadecenoic acid was prepared from castor oil and purified as described It was methylated by refluxing for two hours with four times its weight of methanol containing 1 per cent sulphuric acid During this process a partial isomerization giving rise to 12-oxo-*trans* 10 octadecenoate became evident The mixture was taken up in ether and washed with water, with 25 per cent potassium carbonate and with four portions of water The ethereal solution was dried with sodium sulphate and the solvent was evaporated The remaining oil was dissolved by cautious heating in 30 parts (v/w) of methanol containing 5 parts (w/w) of urea The mixture was allowed to stand at room temperature until the following day when the urea inclusion compound containing the methyl 12 oxo-*trans* 10 octadecenoate was filtered off The filtrate containing the unisomerized fraction was diluted with water, and the lipids were extracted with petroleum ether The extract was washed with water and dried and the solvent was evaporated The remaining oil was isomerized by refluxing for 10 minutes with 10 parts (v/w) of acetic acid containing 0 1 per cent (v/v) methane sulphonic acid The reaction mixture was poured into ice water and the esters were extracted with petroleum ether The extract was washed with successive portions of water and dried and the solvent was evaporated The product was dissolved in 30 ml methanol containing 5 g urea per g lipid and

f ltered the next dav Methyl 12 oxo *trans* 10 octadecenoate was concentrated from the comb ned harvests of crystals of urea inclus on compound by tl e add t on of water and extract on w th ether The ethereal solut on was wasl ed and dr ed and the ether was evaporated The ind dual steps of the preparat on were controlled by thin layer chromatography (s l ca gel G sol ent system pe troleum ether—ether—acet c ac d 90 10 1) and by determinat on of the molar ext nct on coeff c ent at 224 nm of solut ons in ethanol

F nal purif cat on was attempted by column chromatography on s l c c ac d (K eselgel HR reinst nach Stahl Merck) A small amount of lesser polar substances was separated by elution with petroleum ether conta ning 1 per cent ether The methyl 12 oxo-*trans* 10 octadecenoate was then elu ed with 10 per cent ether n petroleum ether and the sol ent was evaporated Th n layer chromatography of the ester revealed the presence of a slightly faster migrating contam nant A small quant ty of this substance could be prepared by repea ed chromatography us ng 1 3 per cent ether in petroleum ether as eluent

The contaminant was ident f ed by a comb na t on of nfrared and nuclear magnet c resonance spectroscopy as methyl 12 oxo *trans* 9 octadecenoate but no su table method for ts separat on on a preparat ve scale was found The content of the contam nant was est mated at 15 per cent

Methyl hydroxyoctadecad enoate was prepared from methyl l noleate hydroperox de by reduct on v th d methyl sulph de It proved to be essent ally pure by th n layer chromatography

Ole c ac d methyl es er (approx ma ely 99 per cent pure) 7 12 d methylbenz[α] anthracene (DMBA) and croton o l were from S gma Che mical Company St Louis M ssouri

The test substances were adm nis ered as solu t ons n acetone (pro analys Merck) A concen tration of 20 per cent (v/v) was chosen for the fatty acid me hyl es ers wh ch was found in pre l m nary experiments to be well tolera ed At the same t me these tes s showed that a 20 per cent solution of methyl oxooctadecenoate n alcohol or acetone was able to provoke a d stinct ep dermal hyperplasia after topical applica ion 3 t mes weekly for 5 weeks Th s short term effect also seen after a preced ng in t at on w th DMBA was weaker than that seen after croton o l It was not observed af er the appl cation of veh cle alone

All solut ons were freshly prepa ed once a month and kept at 20° C between appl ca ons

An mals Male and female nb ed ST/a m ce orig nat ng f om the F b ger Labo atory and bred in our labora ory ere housed n macrolone cages and fed a pelleted s ock d et (Ros ock mixture Korn og Foders of Kompagn e Copenhagen) wi h water *ad l b um* Prel m nary experiments using ha r colouring *Borum* 1954) and sk n b op s es showed that the second rest ng phase of the ha r cycle s arted at about 45 days of age Conse quently the appl cation of test substances was start ed when the an mals were 6½ to 8 weeks old Males then we ghed about 20 g females about 15 g

Experimental procedures All solut ons were dropped onto the unprepared nterscapular sk n w th a syr nge p pette adjusted to del ver 0 05 ml The animals were treated in the per od from 8 to 9 a m One control group was left untreated (Group 1)

The fatty ac d methyl esters were tested as com plete carc nogens by appl cat on 3 t mes weekly (Groups 2-4)

The esters were also tested as promoters after prev ous in t at on w th DMBA The n t ating dose of DMBA chosen (50 m crograms) was sup posed to be subeffective Th s was controlled by means of a group of m ce g ven th s amount as sole treatment (Group 5) Promot on w th fatty ac d methyl es ers was started two weeks after the in t at on w th DMBA the esters were g ven 3 times weekly (Groups 7 9) Croton o l served as pos t ve promoter control and in t ally was also g ven 3 t mes weekly but certa n s gns of general toxic ty prompted after about 4 months a re duction to two weekly appl cat ons (Group 10) A further pos tive control group was given a s ngle dose of 250 m crograms of DMBA (Group 6)

The an mals were nspected once a week Ben gn sk n tumours (pap llomas) were coun ed n the cumulat ve totals when at least 1 mm in d ameter and observed at three success ve inspec t ons

Sk n appl cat on was d scon nued after 1 year and the an mals were observed for another year or until spontaneous death An mals in d stressed tumourous s.age were sacr f ced (F gure 2)

Extract ons of the sk n surface l p ds were made w th acetone as g ven by *Glavind & Chr stensen* (1969) The solut on was f ltered evaporated to dryness and we ghed The extract was d ssolved n a su table amount of hexane (n Hexan fur d e Spektroskop e Merck) An al quot part was d lu ed wi h the same solvent to g ve a concentrat on of about 0 02 mg/ml The spectrum from 210 240 nm vas measured in a Beckman Spectrophotome er DU w th 10 mm quartz cells Further al quot parts of the extract were used for th n layer chromatography wh ch was made on s l cagel G us ng tl e sol ent sys em petroleum ether–ethyl ether acet c ac d 80 20 1 and for the de erm nat on of perox des

Autopsy was performed on all m ce and the in erscapular sk n left k dney spleen l er heart and lungs were rout nely proces ed for h stological study Pap llomas were conf rmed h stologically if not regressed before the dea h of the an mal Other t ssue was removed as well when grossly abnormal

TABLE 1 *Skin Tumour Induction in ST/a Mice by Skin Application of Some Fatty Acid Methyl Esters as Carcinogens and as Promoters Cumulative Results after 18 Months*

Group	Treatment Initiator (dose/mouse)	Promoter	No of mice	Survivors at 18 months	Cumulative no of mice with skin tumours Benign	Malignant	Days to first skin tumour	Papillomas per tumour bearing mouse
1	None	None	25	12	0	0	–	–
2	None	MO*	35	14	3	0	318	1 3
3	None	MOO§	35	17	9	0	3?0	1 9
4	None	MHO†	22	11	2	0	373	1 0
5	DMBA$ 50 μg	None	20	9	0	1	254	–
6	DMBA$ 250 μg	None	20	0	14	0	59	3 4
7	DMBA$ 50 μg	MO*	30	1	8	7	170	1 5
8	DMBA$ 50 μg	MOO§	30	3	20	6	80	2 9
9	DMBA$ 50 μg	MHO†	23	9	18	2	146	2 9
10	DMBA$ 50 μg	COþ	20	1	15	3	41	3 6

* Methyl oleate, 20 per cent (v/v) in acetone, 0 05 ml 3 times weekly
§ Methyl 12 oxo *trans* 10 octadecenoate, 20 per cent (v/v) in acetone, 0 05 ml 3 times weekly
† Methyl hydroxyoctadecadienoate, 20 per cent (v/v) in acetone, 0 05 ml 3 times weekly
$ 7,12 Dimethylbenz[α]anthracene in 0 05 ml acetone, single application
þ Croton oil, 0 5 per cent (w/v) in acetone, 0 05 ml twice weekly

RESULTS

The results of the tests of methyl oleate, methyl 12 oxo *trans* 10-octadecenoate, and methyl hydroxyoctadecadienoate for carcinogenic properties by skin application to mice are shown in Tables 1 and 2 and Figures 5 and 6

An assessment of skin tumour induction based on the cumulative results after 18 months (Table 1) has been made because, in the groups treated with DMBA as initiator, virtually no mice survived after the complete experimental period of 24 months (Table 2) The results after two years are presented in Figure 5 The animal groups are divided according to sex and within each group the total number of mice with skin tumours is related to the number of mice surviving the time of appearance of the first papilloma The total of malignant skin tumours could on a histomorphological basis, be subdivided into 20 squamous-cell carcinomas and 9 sarcomas, chiefly of fibroblastic type This distinction may, however, be illusory, as some of the carcinomas in low differentiated areas assumed a sarcoma like appearance (Figures 3 and 4)

The occurrence of malignant lymphoma in the groups is shown in Table 2, the results for male and female mice are presented separately Figure 6 shows a comparison between the mean survival times of mice with lymphoma and of mice with malignant skin tumours The lymphomas were generalized or *localized* and were made up of proliferating lymphocytes, plasma cells or reticulum cells With a few exceptions, the treated skin area in mice with generalized lymphoma was heavily infiltrated with the neoplastic cells For comparison skin was removed from the hindmost part of the back and from the left inguinal region of 15 of these animals, in these regions slight or no leukaemic infiltration was found It was the general impression that severe lymphomatous

TABLE 2 *Lymphoma Induction in Male and Female ST/a Mice by Skin Application of Some Fatty Acid Methyl Esters as Carcinogens and as Promoters Cumulative Results after 24 Months*

Group	Treatment Initiator (dose/mouse)	Promoter	No of mice ♂	No of mice ♀	Survivors at 24 months ♂	Survivors at 24 months ♀	Cumulative no of mice with lymphoma ♂	Cumulative no of mice with lymphoma ♀	Days to first death with lymphoma ♂	Days to first death with lymphoma ♀
1	None	None	15	10	0	8	0	4	–	712
2	None	MO*	20	15	0	9	1	8	406	426
3	None	MOO§	20	15	0	1	1	7	549	491
4	None	MHO†	8	14	0	5	0	4	–	538
5	DMBA$ 50 µg	None	10	10	0	4	0	2	–	667
6	DMBA$ 250 µg	None	10	10	0	0	8	10	220	121
7	DMBA$ 50 µg	MO*	19	11	0	0	1	1	265	239
8	DMBA$ 50 µg	MOO§	20	10	0	0	10	1	127	565
9	DMBA$ 50 µg	MHO†	8	15	0	1	0	4	–	338
10	DMBA$ 50 µg	COþ	10	10	0	0	6	7	128	114

* Methyl oleate 20 per cent (v/v) in acetone 0 05 ml 3 times weekly
§ Methyl 12 oxo-*trans* 10 octadecenoate 20 per cent (v/v) in acetone 0 05 ml 3 times weekly
† Methyl hydroxyoctadecadienoate 20 per cent (v/v) in acetone 0 05 ml 3 times weekly
$ 7 12 Dimethylbenz[α]anthracene in 0 05 ml acetone, single application
þ Croton oil 0 5 per cent (w/v) in acetone 0 05 ml twice weekly

infiltration in the interscapular region might suppress epidermal hyperplasia and the growth of papillomas in this region

Other tumours are not tabulated We found no correlation with treatment, but a positive correlation with survival time of the animals Most of the tumours were adenomas of various organs, and furthermore some papillomas of the forestomach were seen One animal was killed by an abdominal liposarcoma.

Amyloidosis was diagnosed when primarily observed in hematoxylin eosin stained tissue sections i e, when deposits were conspicuous The diagnosis was confirmed by staining with alkaline Congo red We found no correlation between the occurrence of amyloid and skin cancers or lymphomas Twelve of the 13 observed cases of amyloidosis were however, seen in male mice and males of the ST/a strain show a high fighting activity Most probably the development of amyloid was correlated with wounding and stress in submissive male mice (*Page* & *Glenner* 1972)

Skin surface lipids of an extra group of mice treated for two months with methyl



Fig 2 Advanced skin cancer of typical appearance This tumour was induced in the female mouse by treatment for 11½ months with croton oil 0 5 per cent in acetone after initiation with dimethylbenz anthracene

9*



Fig 3 Same tumour as in figure 2 The photomicrograph shows squamous cell carcinoma developing a sarcoma-like picture (125 ×)

oleate as in Group 2 were extracted on a day following the application of the ester The weight of lipid per animal was, in three experiments, 22 9, 14 6 and 20 6 mg, in comparison with 25 9, 15 0 and 20 2 mg extracted from a group of untreated mice No qualitative difference could be found between the lipids of the two groups when examined by thin-layer chromatography or UV-spectroscopy In particular, no evidence for the presence of oxooctadecenoate in the group dropped with methyl oleate could be found, and the peroxide estimates gave 9, 17 and 12 μequiv /g against 16, 25 and 13 in untreated mice

DISCUSSION

Elaborate studies on carcinogenic properties of oxygen-containing derivatives of the physiological unsaturated fatty acids in warm-blooded animals have not been performed *Van Duuren et al* (1965) in their extensive testing of epoxides, lactones and peroxy compounds found no carcinogenic activity of methyl oleate hydroperoxide and methyl linoleate hydroperoxide towards mouse skin, but concentrations were low and a survey for promoting ability was not included in the tests Aqueous extracts of UV-irradiated linolenic acid, probably containing peroxides and carbonyl compounds, may promote liver carcinogenesis (*Mulay et al* 1969)

In the present studies tumour-promoting ability of the selected fatty acid esters could be demonstrated within 1 year (*Arffmann & Glavind* 1971).

Cumulative results after 18 and 24 months of observation definitely showed methyl 12-oxo-*trans*-10 octadecenoate and methyl hydroxyoctadecadienoate to be rather potent promoters of skin papillomas in mice The



Fig 4 High magnification of the sarcoma like, anaplastic area in figure 3 (500 ×)

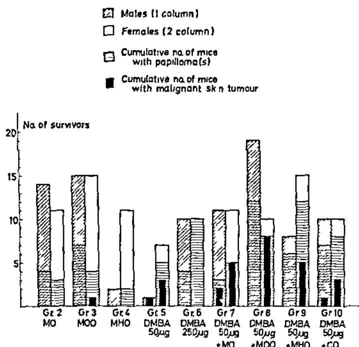


Fig 5 Skin tumour incidence in male and female ST/a mice after topical application of DMBA as initiator or as complete carcinogen and some fatty acid methyl esters as promoters or as complete carcinogens Cumulative results after 24 months
The cumulative number of mice with skin tumours is related to the number of mice surviving the first appearance of a papilloma in the group
MO Methyl oleate, 20 per cent (v/v) in acetone, 0 05 ml 3 times weekly
MOO Methyl 12-oxo-*trans* 10-octadecenoate, 20 per cent (v/v) in acetone, 0 05 ml 3 times weekly
MHO Methyl hydroxyoctadecadienoate, 20 per cent (v/v) in acetone, 0 05 ml 3 times weekly
DMBA· 7,12 Dimethylbenz[α]anthracene in 0 05 ml acetone, single application
CO Croton oil, 0 5 per cent (w/v) in acetone, 0 05 ml twice weekly

effect was similar to that of croton oil, but croton oil was given in a much lower dose and yet the induction time required was significantly shorter, and the resulting number of papillomas per mouse was higher

Methyl oxooctadecenoate gave a shorter induction period and a greater number of malignant skin tumours than hydroxyoctadecadienoate and presumably has the strongest activity of the two compounds (Table 1) Methyl oleate had some papilloma promoting potency, but all parameters designated this effect as weak compared to that of the other esters In contrast, it had a relatively high potency as skin cancer promoter, comparable to that of oxooctadecenoate

It appears from Figure 5 that within the last 6 months of observation a growing incidence of both benign and malignant skin tumours occurred among the few survivors of group 5 This result shows that, after a protracted latency period, even the low dose of DMBA was effective to the mouse skin

No skin tumours appeared in the untreated control group 1, but skin tumours were induced by all the three fatty acid methyl esters, even without previous initiation (Table 1) Methyl oxooctadecenoate was the most potent agent, especially in males (Figure 5), and one sarcoma was identified at necropsy after 688 days

The selected fatty acid esters also promoted the induction of malignant lymphomas (Table 2) The spontaneous incidence of lympho-

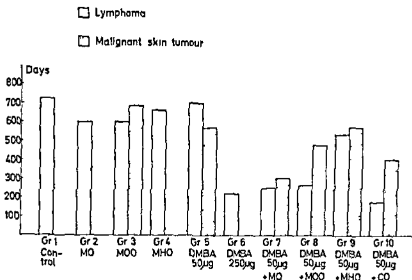


Fig 6 Mean survival time of lymphoma bearing ST/a mice and mice with malignant skin tumour The mice were treated by topical application of DMBA as initiator or as complete carcinogen and some fatty acid methyl esters as promoters or as complete carcinogens

MO Methyl oleate 20 per cent (v/v) in acetone 0 05 ml 3 times weekly
MOO Methyl 12 oxo *trans* 10 octadecenoate, 20 per cent (v/v) in acetone, 0 05 ml 3 times weekly
MHO Methyl hydroxyoctadecadienoate, 20 per cent (v/v) in acetone 0 05 ml 3 times weekly
DMBA 7,12 Dimethylbenz[α]anthracene in 0 05 ml acetone, single application
CO Croton oil 0 5 per cent (w/v) in acetone 0 05 ml twice weekly

ma in our experimental animals can be seen from the results of the untreated group 1, it is not exceeded in control group 5, nor in groups 4, 7 and 9 A high incidence was found in the positive control groups, and also oxooctadecenoate was found to possess a fairly high lymphoma promoting effect (Group 8)

Competition between skin tumours and lymphoma will influence the neoplastic response and complicate the quantitative comparison of the groups Generally the mean survival time of mice with lymphoma was shorter than that of mice with malignant skin tumour (Figure 6) Consequently the low incidence of lymphoma in the females of group 8 is the background for the ensuing high incidence of malignant skin tumours And, conversely deaths from lymphoma within 6 to 12 months reduced the chances of developing malignant skin tumours in groups 6 and 10

A more elaborate pattern appears when the lymphoma producing response is compared by the use of more parameters When the latency period to the first death with lymphoma is used, the response for oxooctadecenoate as promoter is similar to that of croton oil or to the effect of a single dose of 250 micrograms of DMBA

The mean survival time of lymphoma bearing mice was markedly reduced in the groups receiving DMBA in a single effective dose or in a subeffective dose combined with one of the fatty acid esters or croton oil as promoter (Figure 6) When this parameter is used hydroxyoctadecadienoate is the weakest lymphoma promoter

When all three parameters are taken into account, the results show that methyl oxooctadecenoate is a moderately active lymphoma promoter The oleate and hydroxyoctadecadienoate are only weakly active Methyl oxooctadecenoate and methyl oleate also showed some activity as complete carcinogens When tested without previous initiation

with DMBA both substances produced an increased incidence of lymphoma and a shorter induction period

A difference in susceptibility of the two sexes was observed (Figure 5) Females were more susceptible to the induction of skin tu-mours both by DMBA and by a subeffective dose of DMBA followed by a promoter With the exception mentioned above (Group 8), also lymphomas were more easily induced in females (Table 2)

When a comprehensive evaluation of the carcinogenic properties of the three fatty acid esters is made and both the skin tumour and the lymphoma inducing properties are taken into consideration methyl 12 oxo *trans* 10 oc tadecenoate appears to be the most potent compound It contained 15 per cent of a con taminant, the closely related methyl 12 oxo-*trans* 9 octadecenoate, but no special reason suggests that the carcinogenic properties are due to the compound which accounted for the smallest amount and represented the slightest molecular alteration in comparison with, e g the newt negative ester of rici noleic acid (12 hydroxy *cis* 9 octadecenoic acid) The tests for methyl oleate and hydro xyoctadecadienoate gave less conclusive re sults Most convincing was the high activity of methyl hydroxyoctadecadienoate as a pro-moter of skin papilloma induction

The chemical structures of methyl 12 oxo-*trans* 10 octadecenoate and hydroxyoctadeca dienoate both contain an oxygen containing group in conjugation with a double bond In previous papers we noticed the possible im portance of conjugation of double bonds in the fatty acid molecule and of substitution in α position at that time demonstrated in newts (*Glavind* & *Arffmann* 1968, 1970) The pre sent studies confirm the usefulness of the newt test as a short term test for preliminary screening of carcinogens In their carcinoge nicity assays of epoxides *Van Duuren et al* (1967) found that conjugation of the epoxide to a double bond or aromatic ring resulted in carcinogenic activity The observations of *Kato et al* (1971) that the antitumour ac tivity of fatty acids depended upon the num ber of carbon atoms in the chain and the de-gree and position of the unsaturation, may be considered in this context

A number of skin cancers and some lymph omas were induced by methyl oleate The re sult is remarkable as oleic esters are ubiqui tous chemical components of the organism On the other hand, *Twort* & *Twort* (1939) con vincingly demonstrated the promoting effect of oleic acid, also for malignant skin tumours Later *Holsti* (1959) confirmed the tumour promoting properties of oleic acid although within the experimental period of 31 weeks, only papillomas were seen

Methyl oleate hydroperoxide and methyl oxooctadecenoate can be formed from methyl oleate by autoxidation and related processes (*Glavind* & *Arffmann* 1970), and a rapid autoxidation of skin surface lipids is known to take place (*Glavind* & *Christensen* 1967) A possible explanation for the carcinogenic properties of methyl oleate may be found in its conversion to these products following the application to the skin

In order to investigate this possibility, we examined the skin surface lipids of a group of mice dropped for two months with methyl oleate and a negative control group We found, however, no evidence for the presence of the conversion products of methyl oleate mentioned above The weight of the skin sur face lipids was not elevated in the group dropped the day before with 10 μl methyl oleate dissolved in acetone This might sug gest a fairly rapid absorption of the ester through the skin in accordance with the sy-stemic effects observed

Further studies on the effects of altered fatty acids will have to be made to elucidate the still obscure relation between chemical constitution and carcinogenic properties and to decide whether chemical change in the body of the substances applied is prerequisite to the formation of specific oncogenic meta bolites

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The authors wish to express their thanks to Dr *J Kieler* The Fibiger Laboratory for his advice on the planning of the experiments to Professor *Chr Pedersen* Department of Organic Chemistry,

Polytechnic Institute, for recording nuclear magnetic resonance spectra of the oxo fatty acid esters, and to Dr H O Bang, Department of Clinical Chemistry, Aalborg Hospital North, for the determination of peroxides in the skin lipid extracts

The work was supported by grants from *Miss Ane Martinussens Foundation*, Aalborg, and the *Danish Government Fund for Scientific and Industrial Research*

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DATING OF THE HUMAN CORPUS LUTEUM OF MENSTRUATION USING HISTOLOGICAL PARAMETERS

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The aim of this investigation has been to examine which histological parameters are of value in the dating of the corpus luteum of menstruation Further to express these parameters in a graphical form which is of practical use for a histological dating The material comprises histological specimens of the ovaries from 24 women selected according to strongly limiting criteria The following histological parameters have been found useful for the dating 1) Extravasated blood and capillaries in the granulosa cell layer 2) The size of the granulosa cells 3) Degeneration of the granulosa cells 4) Invasion of theca cells into the granulosa cell layer 5) Organization of the central cavity These parameters have been graded semiquantitatively, and the results have been the basis of the curves prepared by electronic data processing of the observations Finally some indications for histological dating of the corpus luteum of menstruation are mentioned

The cyclic morphologic changes which the corpus luteum of menstruation undergoes have been the object of several studies both in animals and humans The most important, essential work in this field was carried out several years ago An example is *Corner's* studies on the corpus luteum of menstruation in monkeys (e g 4) It seems to be *Meyer* (e g 6,7) who has performed the fundamental more extensive histological studies of the corpus luteum *Breuer* in 1942 states "It is to Dr Meyer, that we are all indebted for the essential work on the corpus luteum (1)

Breuer's own papers (1, 2, 3) represent the studies which we chiefly will refer to, as he has given a more detailed description of the different phases in cyclic morphologic changes which take place in the human corpus luteum

Received 19 ix 73 Accepted 19 ix 73

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In some cases it is of importance to be able to date a corpus luteum of menstruation on histological examination An accurate dating is, however, difficult on the basis of the histological studies up till now

The aim of this work has been

A) to examine which histological parameters are of value in the dating of the corpus luteum of menstruation
B) to express these parameters in a graphical form which is of practical use for histological dating

MATERIAL AND METHODS

The material was highly selected and consisted of 24 normally menstruating women aged 22 to 45 years For the whole group the length of the cycles ranged from 26 to 30 days but for the individual woman the variation in the length of the cycle was only ± one day

All 24 women underwent laparotomy during the second half of the cycle, and the indication for the

Polytechnic Institute, for recording nuclear magnetic resonance spectra of the oxo fatty acid esters, and to Dr *H O Bang*, Department of Clinical Chemistry, Aalborg Hospital North for the determination of peroxides in the skin lipid extracts

The work was supported by grants from *Miss Ane Martinussens Foundation*, Aalborg, and the *Danish Government Fund for Scientific and Industrial Research*

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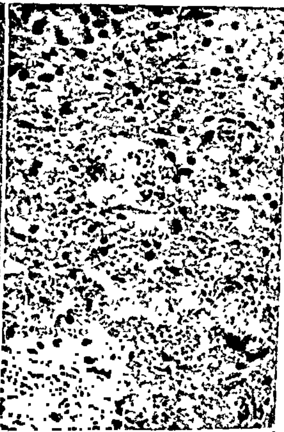




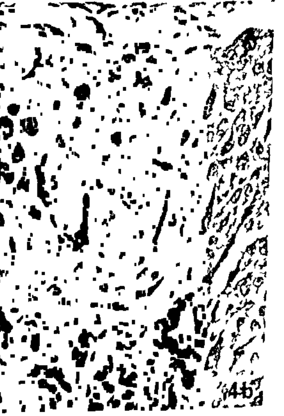

2a


2b









and vessels are brought along, and towards the end of the cycle a degeneration of the theca cells takes place with transformation into connective tissue, and the boundary to the granulosa cells becomes blurred

Organization of central cavity Already three days after the ovulation a few fibroblasts may sometimes be seen in the blood filled cavity facing the granulosa cell layer The number of fibroblasts increases, and collagen fibrils are laid down The extent of the organization has been evaluated semiquantitatively, and consideration has been taken to the size of the cavity About the 9th day pronounced changes with complete organization of narrow cavities (Fig 4a, b) and pronounced organization of the outer zones of large cavities are seen

Graphical Representation of the Histological Parameters

Fig 7 shows the semiquantitative evaluation of the histological parameters used The granulosa cells are classified as small, medium, larger For the remaining four parameters the grades 0, slight, moderate and marked, have been used

The graphical representation of the observations has been produced using a least squares curve fitting algorithm on a computer

For the observations on blood and capillaries rational algebraic fractions have been introduced in the algorithm For the remaining four parameters an exponential regression forms the basis for the electronic data processing

DISCUSSION

As mentioned in the introduction the most comparable work has been published by *Brewer et al* (1, 2 3) These investigators have examined the human corpus luteum both during the normal menstrual cycle and during pregnancy In addition they have compared the histological changes in the corpus luteum of menstruation and in the endometrium

Brewer (1), just as e g *Frank* (5), divide the development of the corpus luteum of menstruation into four stages proliferation stage, vascularization stage, mature or blossom stage, and regression stage This division is solely based on the morphologic changes The proliferation stage comprises the first few days, the vascularization stage the following 5–6 days, then follows the mature stage, and the regression stage is reckoned from the commencement of the menstruation It is, however, not so much the division into stages *Brewer* concerns himself with, but above all the histological changes during the period A comparison between *Brewer's* histological findings (1) and our own observations shows good agreement

Own material We have considered the strong selection of our material necessary, even though the number of patients thus has become modest It is the general opinion, that the interval between ovulation and the commencement of menstruation is rather constant and can for most women be set at fourteen days This has been made the basis for calculating the age of the corpus luteum. The distribution of our material on the fourteen days is not optimal, as especially the first and last days are sparingly represented

Fig 3a+b Day 7 Marked amount of extravasated blood and capillaries between the medium sized granulosa cells H E × 38 (a) × 240 (b)

Fig 4a+b Day 9 No extravasated blood and no functioning capillaries between the larger granulosa cells Marked invasion of theca cells and marked organization of the central cavity H E, × 38 (a) × 240 (b)

Fig 5a+b Day 11 Granulosa cells showing moderate degeneration, and moderate fibroblast proliferation in the granulosa cell layer Marked organization of the central cavity H E, × 38 (a) × 240 (b)

Fig 6a+b Day 13 Marked degeneration of granulosa cells and marked fibroblast proliferation No evident demarcation between the granulosa cell layer and the theca cells H E, × 38 (a) × 240 (b)

5a

5b

6a

6b

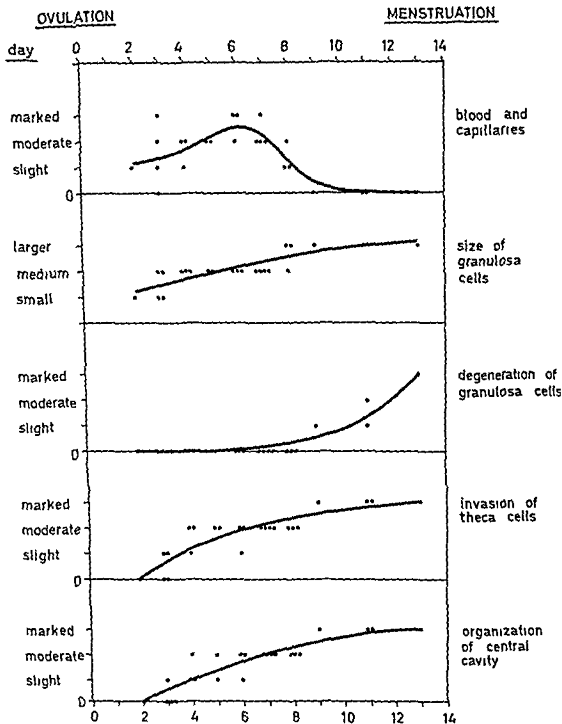


Fig 7 Dating of the human corpus luteum of menstruation Approximate relationship of useful histological parameters

In the semiquantitative evaluation of the histological parameters it has not been found reasonable to differentiate between more than the few grades which are shown in Fig 7 It will appear, that there in several places is some variation in the parameters in women on the same day Thus, there seems to be a considerable difference in the degree of vascularization of the granulosa cell layer during the first days All four degrees of vascularization are represented on the third day

The individual observations are marked in Fig 7 The full drawn curves have been calculated by electronic data processing on all available observations

Indications for dating of the corpus luteum of menstruation In the majority of pathology departments which serve gynaecologic departments it is a routine task to date the endometrium on the basis of a series of histological parameters This is in practice performed in the easiest manner using a series of curves of the changes in the histological parameters during the cycle, (9)

Sometimes the clinicians will be interested in an assessment of the corpus luteum It may e g be a case of differentiating between a corpus luteum of menstruation and a corpus luteum of pregnancy which in the majority of cases poses no problem But, it may also be a case of dating a corpus luteum of menstruation which, e g, may be indicated in certain forensic cases or in cases where an evaluation of the effect of different hormone preparations on the function of corpus luteum is needed

In analogy with the use of curves for dating the endometrium the curves presented in this paper should contribute to a more rapid and more reliable histological dating of the corpus luteum of menstruation

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The company *Christian Rovsing A/S* has most kindly performed the electronic data processing

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Acta path microbiol scand Section A 82, 145–152 1974

VALUE OF FREE METAPHASE CELL PREPARATIONS IN CYTOPHOTOMETRIC STUDY OF EXPERIMENTAL SKIN CARCINOGENESIS

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The following topics are presented 1) an enzymatic method for selective separation of hyperplastic epidermis from hair bearing mice and its dissociation into free cell preparations rich in metaphase cells, 2) a quantitative cytochemical test (cytofluorometric determination of Feulgen DNA) on the retainment of Feulgen reactivity by the dissociated cells and 3) an example of the application of the method to investigating chemical skin carcinogenesis It is possible by this method to assay cytophotometrically the DNA of epidermal cells at the same phase of the generative cycle It turned out that the DNA content of epidermal meta phase cells in the group of mice treated with a carcinogen and mechanical irritation (3 weeks) was significantly greater than the corresponding value of the group which received only acetone and mechanical irritation This seems to suggest that a treatment with a carcinogen causes fairly early changes in the nuclear material of the germinative tissue The method described offers new possibilities for quantitative cytochemical characterization of the nuclear changes associated with experimental carcinogenesis because it eliminates the sources of error normally present in section preparations and the problems of interpretation caused by the cell cycle, and permits the use of cytofluorometry a method considerably more sensitive than absorption cytophotometry

When quantitative cytochemical methods were first applied to the study of nuclear changes associated with chemical skin carcinogenesis in our laboratory, it turned out that the quality of the skin preparation used for the cytophotometric determination of the DNA contents of individual epidermal cells was crucially important The sources of error present in section preparations (partial overlapping and cutting off of nuclei) motivated the development of a new method, which can be used to separate the epidermis of a hair-bearing mouse from the dermis selectively without adnexal contamination, and to dissociate it into free cell preparations whose cells retain their morphology and display no changes in Feulgen reactivity (1) The separation consists of two phases digestion in α-chymotrypsin, and removal of the epidermis which has lost its coherence with adhesive tape The free cell method improves the accuracy of measurement by eliminating the sources of error involved in section preparations and by permitting the use of cytofluorometry, which is a far more sensitive

Received 4 vi 73 Accepted 21 ix 73

method for measuring the DNA contents of single cells than absorption cytophotometry (3)

When the cytophotometric method is used in studies of the DNA changes of skin carcinogenesis, there is also the additional difficulty that the DNA values of the S phase nuclei which occur in a rapidly proliferating tissue may overshadow possible small aneuploid cell populations (5, 11) One way of eliminating this disadvantage would be to carry out the measurements on cells at the same phase of the generative cycle The metaphase offers a natural opportunity for this

The present work is concerned with the application of the above enzymatic method by which free cell preparations rich in metaphases may be obtained from hyperplastic epidermis of hair-bearing mice, with a cytofluorometric test of the retainment of Feulgen reactivity by the dissociated cells, and an example of the applicability of the method in investigations of chemical skin carcinogenesis

MATERIALS AND METHODS

Induction of Epidermal Hyperplasia

Male NMRI mice aged 18 weeks were used in the experiment They were divided into two groups of 25 mice which were given the following weekly treatment for 3 weeks (the days of the week are numbered)

Group A

1st day The dorsal hair was removed with a razor, and one drop of 0 2 per cent DMBA (9 10 dimethyl 1 2 benzanthrancene)–acetone solution was applied to the shaven area

3rd and 4th day The manipulation area was irritated mechanically by rubbing it lightly with grade 0000 sandpaper at a force as constant as possible

Group B

The treatment was otherwise identical with that given to group A but a drop of acetone was applied instead of the DMBA solution

Separation of Epidermis and Preparation of Smears

On the first day of the fourth week the animals were killed In the morning each mouse was given 0 12 mg of colchicine in physiological saline intraperitoneally After 4 hours the mice were killed by cervical dislocation under ether anaesthesia The depilated (1) dorsal skin was removed by cutting with scissors and stripping off the piece of skin, including the panniculus carnosus, after which the skin piece was stretched on a filter paper, epidermis upwards A piece of skin measuring 30 mm × 30 mm and located symmetrically on the middle line with its front edge at the scapular level was cut off to be used in the experiment The filter paper adherent to the skin was allowed to remain and the skin piece was attached to it with four tacks at the corners This made the skin piece firm and easier to handle

Enzyme treatment of the skin Fifteen mice of each group were used for this purpose The enzyme preparation employed was 0 5 per cent (w/v) α chymotrypsin solution (α Chymotrypsin Nr 2307 45 mU/mg E Merck AG, Darmstadt) made in Hanks' balanced saline solution Incubation took place in 25 ml of the enzyme solution in a petri dish in a water bath at +37±0 1° C The remaining 10 animals in each group were used for histological controls of hyperplasia

Mechanical separation of the epidermal components After 3 hours of enzyme incubation the epidermis was separated with adhesive tape and forceps from an area of the breadth of the tape (details of the procedure have been described earlier (1)) It turned out that hyperplastic epidermis could not be removed as easily and evenly as normal epidermis (1), but with the help of forceps it was possible to remove most of the epidermis along the incision at the edge of the tape, and abundant material for free cell preparations was thus obtained

Free cell preparations The free cell preparations were obtained by scraping off material with the edge of a slide from the back side of the separated epidermis attached to the tape After the removal of epidermis, the remaining piece of skin was cut into 3 parts along the edges of the area with removed epidermis Further material was scraped from the midmost portion to obtain adnexal free cell preparations The lateral portions were used for histological control of the enzyme action

Histological and Cytochemical Techniques

The skin pieces and free cell preparations were fixed in ethanol glacial acetic acid (3 1) for exactly one hour The pieces of tissue were cut into paraffin sections 6 um thick which were stained with haematoxylin eosin Some of the smears were also stained with haematoxylin eosin

For the cytofluorometric determination of DNA the smears were stained with the Feulgen technique Hydrolysis took place in 5 N HCl at +23° C for 40 minutes The fluorochrome employed was Auramine 0 (C I Nr 41000 E Merck AG Darmstadt), which replaces the basic fuchsin of Schiff's reagent (12) *Harleco Fluorescence Mountant* was used as the embedding medium The standard

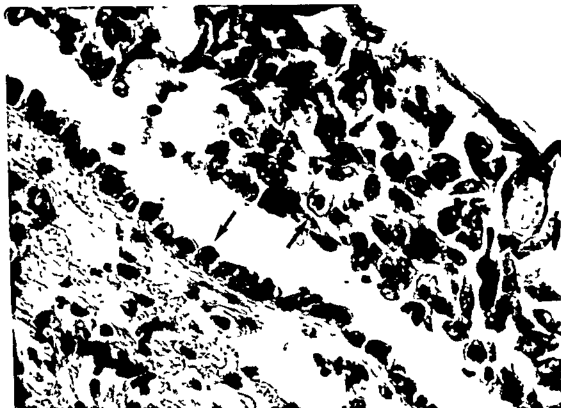


Fig 1 Effect of chymotrypsin digestion on hyperplastic mouse epidermis (DMBA treatment and mechanical irritation for 3 weeks) Separation has been suprabasal and intra epidermal. Metaphase cells (arrows) are numerous in the deeper layers of the separated epidermal tissue and in the basal cell layer Paraffin sections Haematoxylin-eosin 400 ×

material consisted of sperms from one bull which were applied to one end of each free cell preparation measured The sperms had been fixed in the same way as the epidermal cells and were preserved in 70 per cent ethanol (12) The reference material for the diploid DNA content consisted of the peripheral blood leucocytes from 6 untreated mice separated by the buffy coat technique Red cell contamination in these preparations was considerable but not disturbing

Cytofluorometry

The measurements were made with a Leitz MPV photometer connected to an Orthoplan microscope The optical equipment (10) and the procedure of measurement was that described in my previous study (1) 20–30 basal cells in the interphase the morphological criteria have been presented earlier (1)) and the metaphase were measured from each free cell preparation obtained from the epidermis with adhesive tape

Statistical Analysis

The F test was used

RESULTS

Effect of Enzyme Digestion on Hyperplastic Epidermis

Irritation effected epidermal hyperplasia in both groups The hyperplasia in the DMBA group was slightly less regular and besides, intracellular oedema occurred in this group Metaphase cells were numerous in the basal layer and occasionally even above it Enzyme digestion caused the epidermal tissue to be separated suprabasally and intra epidermally (Fig 1) It could be seen from the histological sections that the basal cell layer generally remained attached to the dermis, though at

Fig 2 Same case as in Fig 1 Mechanical separation of the epidermis following chymotrypsin digestion has removed the epidermal cells almost entirely Only part of the basal cells have remained attached to the dermis The coherence of the appendages has been weakened, but the cells have not been detached Paraffin sections Haemotoxylin eosin 250 ×



Fig 3 Free cell preparation obtained from the epidermis separated after chymotrypsin digestion Four metaphase cells in the middle Haematoxylin eosin 1000 × Oil immersion

some points even the basal tissue was separated. Mechanical separation following the enzyme digestion removed the epidermis in such a way that most of the basal layer was also removed (Fig 2) The hair follicles and the sebaceous glands were only removed if the dermal part remaining after the application of adhesive tape was manipulated (Fig 4)

Free Cell Preparations

The free cell preparations were very rich in cells The cell types representing the various

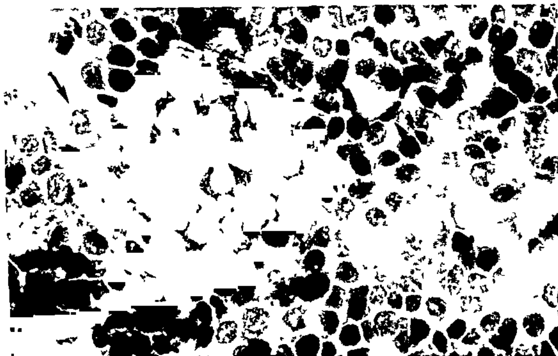


Fig 4 Free cell preparation obta ned by scraping off material with the edge of a sl de from a sk n whose ep dermis has been removed after chymotryps n digest on The occurrence of sebaceous cells (arro vs) in the preparation shows it to be of adnexal or gin Haematoxyl n eos n 250 ×

stages of different ation and the metaphase cells were easy to recognize (Fig 3) The preparations obtained from the epidermis separated by adhesive tape generally con tained no hair follicles or sebaceous gland cells But the preparations made from the dermal part which remained after the ap plication of adhesive tape contained plenty of disintegrated hair follicles and sebaceous gland cells (Fig 4) Spindle shaped cells in dicating dermal contamination were very few

The cells treated according to the Feulgen technique were easily recognizable by the phase contrast method The metaphase cells had retained their morphology perfectly well and the intensity of their nuclear fluorescence was strong (Figs 5 a and b)

Cytofluorometry

The results are given in Figures 6 and 7 The interphase cells of each group had a modal value between the diploid and the tetraplo d values which indicates that a large proportion of the cells measured are in the S phase

The metaphase cells of the group which received only acetone and mechanical irrita tion had a sharp modal value which cor responds quite accurately to the tetraploid value This shows that the cells have ap parently retained their Feulgen reactivity well during the enzyme digestion

In the group which received both DMBA and mechanical irritation the attention is drawn to the broadening of the DNA fre quency histogram of the metaphase cells in the hypertetraplo d direction and the absence of any sharp modal value The average DNA content of the metaphase cells proved to be significantly greater in the DMBA group than in the group which received only acetone and mechanical irritation (50 8±6 2 AU and 54 9±7 7 AU $p < 0\,001$) The number of hypertetraploid cells among the interphase cells



Fig 5 Smear obtained from the ep derm s separated after α chymotrypsin digestion The smear has been Feulgen hydrolysed and sta ned with Sch ff's reagent in which the bas c fuchs n has been replaced w h Auramine O
A) A phase contrast photograph focussed on a metaphase cell in the m ddle 1100 × Oil immers on
B) Same preparation in UV illumination The fluorescence of the me aphase nucleus s very intens e The intensity of the nuclear fluorescence of interphase cells and d fferent ating cells var es

of the DMBA group is too small to warrant any such conclusion that the group might have pathologically high DNA contents

DISCUSSION

As shown in my previous work α chymo trypsin is a better agent for dissociating epidermis into free cell preparations than trypsin as regards both the selectivity and effectiveness of separation and the preserva tion of the nuclear material of ep dermal cells (1) It is not clear to me why the effects on nuclear material are different The reason why α chymotrypsin was chosen after the adverse effect of trypsin on nuclear material had been noted was that α chymotrypsin on the basis of its different chemical point of action and the amino acid composition of the nuclear basic proteins could be expected to have less lytic effects on the nuclear material

The results of the present work showed that α chymotryps n is also well fit for the produc tion of free cell preparations from hyper plastic epidermis Although hyperplastic epi dermis required the enzyme concentration to be raised from 0 25 per cent to 0 5 per cent and the digestion time to be increased from 2 to 3 hours the cells retained their morpho logy well It could also be seen that the epidermal cells retained their Feulgen reac tivity The sharp tetraploid modal value of

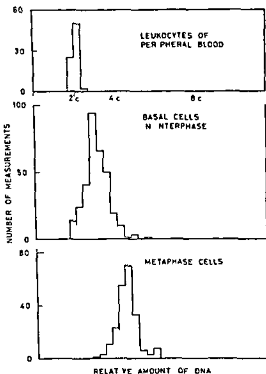
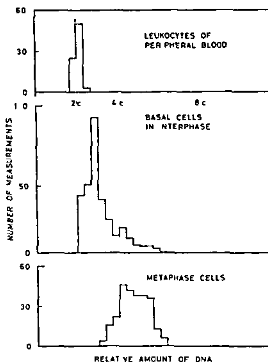


Fig 6



F g 7

Fig 6 and 7 Frequency h stograms of the Feulgen D\A values of ep dermal cells separated by the α chymotryps n method To fac l tate compar son of the h stograms the f g res include the reference l nes drawn to correspond to the d plo d D\A con ent (?c) of the leucocytes and its polyplo d multiples A more detailed explanation of the f gures is given n the text

the metaphase cells obtained from mechanic ally irritated epidermis sho s that it is pos sible to measure the DNA content of epi dermal cells at the same phase of the gene rative cycle by this method

If the method here described is used in investigations into chemical skin carcino genesis it elim nates the sources of error in volved in section preparations and the pro blems of interpretation due to the generative cycle Problems of the latter type include the po s bility that the S phase nuclei may over sl adow potential small aneuplo d cell popula t on in a tissue with rapid proliferation and/or abundant DNA synthes s The cyto pl otometric DNA assays of skin carcino genes s which have been reported in the l terature have not succeeded in revealing changes specific to carcinogenesis Both non specif c irritation and carcinogenesis treat ment have produced roughly sim lar DNA histograms the broadening of the histograms in the tetraploid direction is an indication of abundant S phase cells (e g 5 9 11) This has given rise to a sceptical attitude towards the postulation that the nuclear changes as sociated with experimental mal gnant trans format ons might be described by quantita tive cytochemical methods (4) although *Manoccl a et al* having measured DNA con tents of metaphase cells in precancerous skin changes in man indicated that aneuploid DNA contents m ght be present in germina tive cells (8)

According to the literature on the cyto photometric investigation of experimental

skin carcinogenesis, interphase cells have been measured non-selectively from section preparations During the last few years, a fair amount of evidence has been accumulated which suggests that the carcinogenetic effect may be restricted to a certain phase of cell cycle, and that the cells synthesizing DNA occupy a crucial position (13) Moreover, there are indications that the basal tissue of the epidermis may be germinatively non-homogenous and that only a certain geographically defined portion participate in germinative action (6, 7) If the carcinogenetic effect is restricted to the germinative tissue in particular, it is clear that any non-selective measurement of epidermal cells, even of basal cells alone, inevitably includes "non-germinative" cells which rules out the possible specifically carcinogenetic effects

The present work is concerned with the application of the method in the investigation of potential DNA changes caused by carcinogenetic action It turned out, unfortunately, that the metaphase cells obtained in the free-cell preparations from non hyperplastic epidermis were so few in number that they could not be used as reference material Although the material is small and does not justify any generalizing conclusions, it was interesting to note that the DNA content of metaphase cells was significantly greater in the DMBA group than in the group which received only acetone and mechanical irritation This suggests that even a carcinogenetic treatment of this short duration may have brought about genuine changes in the nuclear material of the ferminative tissue

It is possible that the method here described offers further possibilities for a quantitative cytochemical characterization of the nuclear changes to occur during experimental carcinogenesis, because it eliminates the sources of error implicit in section preparations and the problems of interpretation due to the cell cycle and permits the use of cytofluorometry which is a much more sensitive method than absorption cytophotometry

It is the intention of the author further to apply this method to cytofluorometric determination of the histones of epidermal cells The qualitative results show that histones tolerate well this separation method (2)

This work was supported by a grant from the *Research Foundation of President J K Paasikivi*

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Acta path microbiol scand Section A 82, 153–160 1974

STUDIES ON THE HISTOGENESIS OF EXPERIMENTALLY INDUCED CERVICAL CARCINOMA

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The alterations preceding invasive carcinoma and the development of frank carcinoma have been investigated in 116 mice following intravaginal painting with 3,4 benzpyrene The subsequent epithelial changes were graded in increased order of deviation from the normal, atypia grade I, II, III, questionable invasive and frankly invasive carcinoma. Slight atypia (grade I) was observed following 2 months application of the carcinogen. Atypia grade II occurred after 3 and 4 months' treatment After 5 months, atypia grade II or III, questionable or invasive carcinoma developed Ninety two per cent of the mice with questionable or invasive carcinoma had in addition intra-epithelial atypias with buds Invasion was seen to originate at the tip of epithelial buds with severe cellular atypias The findings strongly suggest an association between epithelial buds with cellular atypias and progression to invasive carcinoma

Carcinoma in situ of the human uterine cervix can be classified histologically into two groups Carcinoma in situ simplex (CISS) is distinguished by the even and smooth epithelial stroma border The other type, carcinoma in situ with buds (CISB), is characterized by irregular buds of epithelium bulging into the stroma In several follow-up studies it has been shown that CISB is more prone to develop into invasive carcinoma than is CISS (*Rubio & Söderberg* 1969–1970)

To understand better the implications of the histological appearance of cervical cancer on the clinical behaviour of the tumour more detailed information about the histogenesis of invasive carcinoma is needed Experimentally, both systemic and local application of a variety of chemical carcinogens have been shown to induce both atypical non invasive changes as well as invasive carcinoma in the cervical epithelium of mice (*Koprowska & Bogacz* 1959, *Dunn* 1963, *Forsberg & Breistein* 1972 and *Rubio & Lagerlöf* 1973) For a study on the histogenesis of cervical carcinoma we have used the method of intravaginal application of 3,4-benzpyrene in mice (*Rubio & Lagerlöf* 1972)

Received 31 viii 73 Accepted 26 ix 73

MATERIALS AND METHODS

A) *Intact Mice Treated with Benzpyrene*

116 virgin C57Bl mice were painted intravaginally twice a week with 1 per cent 3 4 benzpyrene *in acetone* The cervical area was visualized through a paediatric octoscope no 1 with proximal *illumination* A sterile cotton swab of the type used for collecting bacteriological specimens from the nasopharynx was soaked in the 3 4 benzpyrene solution and introduced through the speculum The cervical area was painted with 4 double strokes After the last stroke the applicator and speculum were withdrawn together in order to check that the applicator had passed the distal *ostium* of the speculum

Of the 116 mice in group A, groups of 10 were killed after treatment for 1, 2, 3 and months The remaining 76 were killed after 5 months

B) Spayed Mice Treated with Benzpyrene

Ten virgin C57Bl mice were ovariectomized at the age of 3 months One week later the mice were started on the same treatment as those in group A The animals were killed in groups of 2 at the same time intervals after treatment as those in group A

C) Acetone Treated Controls

In 25 virgin C57Bl mice, acetone was applied to the cervical area by the same technique as that used in groups A and B The animals were killed in groups of 5 at the same time intervals

All experimental and control animals were kept in an air conditioned room and on a standard pellet diet The animals were ear marked to make it possible to follow the cytological picture during carcinogenesis (*Rubio* & *Lagerlof* in preparation) for comparison with the histological pattern in the individual animal

One animal in group A died after treatment for 5 months This animal had a large carcinoma in the cervical area The remaining 150 animals were killed at the end of the alloted survival time (see above) The cervix portio area was removed and fixed in 10 per cent formalin for conventional histological studies Sections were done along the oral caudal axis and stained with H & E and silver stain (*Bloom* & *Fawcett* 1968)



Fig 1 Atypia Grade I (× 400)

Distribution of Atypical Epithelium

The distribution of epithelium with buds was correlated to the total amount of atypical epithelium in sixty sections from 10 consecutive mice with the aid of a calibrated ocular micrometer The 10 mice were taken from the group of animals with epithelial buds with atypia (cf Tables 1 and 2)

RESULTS

Treatment of the cervix of mice with benzpyrene produced cellular atypia in the squamous epithelium The mucosal alterations were more pronounced in the ectocervix than in the cervical canal The epithelial atypias were graded as follows

Atypia grade I Mild atypia with mild nuclear polymorphism and single-cell keratinization with various degrees of nuclear degeneration (Fig 1) Grade I atypia also includes basal cell hyperplasia Cytoplasmic maturation towards the surface was evident Intra epithelial micro abscesses were often present Atypia grade I was observed in 34 mice (21 in the experimental group with intact ovaries (group A) (cf Table 1) in 4 of the spayed animals (group B) and in 9 in the acetone group (group C) (cf Table 2)

Atypia grade II More severe nuclear atypia The nuclei were polymorphic and hyperchromatic Single cell keratinization was also found Irregular buds of squamous epithelium as well as more pronounced inverted papillomatosis with cellular changes of grade II atypia were observed Intercellular bridges were distinct Cytoplasmic maturation towards the surface was still evident although less distinct than in atypia grade I (Fig 2) Mitoses were observed in the intermediate layers Atypia grade II was observed in 33 mice (all in experimental animals with intact ovaries) (Cf Table 1)

TABLE 1 *Classification of 3 4 Ben-pyrene Induced Lesions in the Uterine Cervix of 116 Mice*

Duration of application	HISTOLOGY Normal NB	Normal B	Grade I NB	Grade I B	Grade II NB	Grade II B	Grade III NB	Grade III B	Quest Inv NB	Quest Inv B	Invasion NB	Invasion B	Total
1 month	10												10
2 months	3	1	1	5									10
3 months	1	1		6		2							10
4 months				5		5							10
5 months		6	1	3	2	24	1	13	1 (III)	8 (6 II) (2 III)	17 (5 II) (11 III) (1 ( ))		76
Total	14	8	2	19	2	31	1	13	1	8	17		116

B Denotes buds of epithelium bulging into the stroma
NB Denotes lack of epithelial buds
In brackets the degree of atypia associated with questionable invasion or invasive carcinoma

TABLE 2 *Classification of Lesions in the Uterine Cervix in 10 Ovariectomized Mice after Application of 3 4 Benzpyrene and in 25 Mice after Intravaginal Application of Acetone (in brackets)*

Duration of application	HISTOLOGY Normal NB	Normal B	Grade I NB	Grade I B	Invasion	Total
1 month	2 (5)					2 (5)
2 months	1 (2)	(1)	1	(2)		2 (5)
3 months	(3)			2 (2)		2 (5)
4 months	(1)	(1)		1 (3)	1	2 (5)
5 months	(1)	1 (2)		(2)	1	2 (5)
Total	3 (12)	1 (4)	1	3 (9)	2	10 (25)

B Denotes epithelial buds
NB Denotes lack of epithelial buds

Atypia grade III Nuclear atypia without evidence of cytoplasmic maturation even in cells at the surface of the epithelium Nuclear polymorphism was considerable and hyperchromatism further accentuated Tonofibrils were often absent Mitosis were observed in the superficial layers The nuclear polarity was lost (Fig 3) Atypia grade III was usually associated with epithelial buds Atypia grade III was observed in 14 mice (all in experimental animals with intact ovaries) (Cf Fig 1)

Questionable invasion The cervix of 9 mice (all in experimental group with intact ovaries) contained areas with an indistinct epithelial-stroma border and/or small groups of epithelial cells apparently isolated in the stroma While the presence of micro invasion could not be ruled out in these animals its occurrence could not be proven satisfactorily The investigation of sections stained with Lendrum stain supported the suspicion of micro invasion

Invasive carcinoma The conventional criteria for diagnosing unquestionable invasive carcinoma were applied Invasive carcinoma could be recognized in 19 mice (17 in the experimental group with intact ovaries and 2 in the spayed animals in group B)

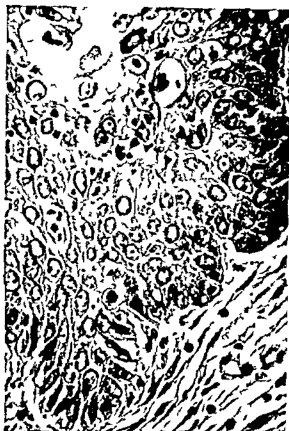


Fig 2 Atypia Grade II (× 400)

Chronological Appearance of Atypical Cervical Epithelium

A) *Intact mice treated with benzpyrene* (Cf Table 1) All 10 animals examined after application of 3,4 benzpyrene for 1 month had a histologically normal cervical epithelium After 2 months treatment, 6 out of 10 animals had atypia grade I (5 of these presented also epithelial buds) After 3 months' treatment, 8 out of 10 mice had grade I or grade II atypia, all 8 with epithelial buds All 10 mice treated for 4 months had grade I or grade II atypia with epithelial buds After 5 months application, 70 of the 76 mice had some degree of atypia—4 grade I, 26 grade II, 14 grade III, 9 had questionable invasive carcinoma, and 17 frankly invasive carcinoma Forty out of the 44 mice with atypia grade I II or III depicted in Fig 1 also had epithelial buds Eight out of the 9 animals with questionable invasion had atypia grade II or III with epithelial buds in the adjacent epithelium and 16 out of the 17 animals with frank invasive carcinoma showed in the vicinity non invasive atypia grade II or III with buds In the remaining mouse the invasion was massive and areas with intra epithelial atypias could not be identified The chronological appearance of cervical atypias is graphically depicted in Fig 4

B) *Spayed animals treated with benzpyrene* (Table 2) The two animals painted for 1 month had a normal cervical epithelium One of the two animals treated for 2 months had grade I atypia without buds After 3 months the two treated animals had grade I atypia with buds After 4 months one animal had grade I atypia with buds and the other had an invasive carcinoma After 5 months one mouse showed invasive carcinoma and the other animal normal epithelium No areas without invasive carcinoma were available for study in the two mice with invasion



Fig 3 Atypia Grade III (× 400)

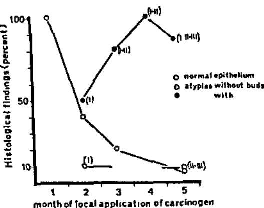


Fig 4 The histological findings in the cervix epithelium of mice after intravaginal application of 3 4 benzpyrene

C) *Acetone treated controls* Among the 25 animals treated with acetone (Table 2) 16 had a normal squamous epithelium without atypia Nine had grade I atypia with buds (two out of five after 2 months, two out of five after 3 months three out of five after 4 months and two out of five after 5 months) More severe degrees of atypia were not observed

Distribution of Atypical Epithelium

In 34 mm atypical epithelium represented in the histological sections from the mice investigated, buds were also present within 27 mm, i e 79 per cent on the average Individual values ranged from 59 to 100 per cent The lowest value was obtained in a mouse with atypia grade II whereas the highest (100 per cent) was observed in mice with atypias grade I, II and III These values do not include epithelial changes with doubtful bud formation

DISCUSSION

Numerous reports discussing the intra epithelial changes preceding invasive carcinoma of the cervix in rodents subjected to local or systemic treatment with hormones and/or carcinogens have appeared in the literature (*Reagan et al* 1955, *Scarpelli* & *von Haam* 1957 *Gardener* 1959 *Koprowska* & *Bogacz* 1959, *Murphy* 1961, *Wentz* 1962, *Dunn* 1963, *Iijima et al* 1964, *Kaminetzky* & *Swerdlow* 1964, *Yong* & *Campbell* 1965, *Kaminetzky* 1966, *Kehar* & *Wahi* 1967, *Graham* 1970 and *Forsberg* & *Breistein* 1972)

However, the possible association between epithelial "buds" and invasive carcinoma has not received much interest

In the present investigation, substantial evidence was provided for the occurrence of irregular epithelial 'buds" associated with cervical atypia induced by local application of a carcinogen, and apparently similar to those characterizing CISB of the human cervix After application of 3,4 benzpyrene for two to five months, 92 per cent, or 57 of 62 animals with non-invasive atypia (cf Table 1) also had epithelial buds The size of these epithelial buds varied In some mice the epithelial buds had an arborescent papillary pattern As is evident in Fig 4, the appearance of epithelial buds and papillary hyperplasia could be related to the duration of the treatment with the carcinogen, the longer the treatment with 3,4 benzpyrene, the higher the incidence of epithelial buds and papillary hyperplasia of the epithelium The same holds for cellular atypia After two and three months' treatment with 3,4 benzpyrene, only slight or moderate atypia were observed Grade III atypia was found only after treatment for five months It should be pointed out that the majority of these (13 of 14 mice with atypia grade III in Table 1) also had epithelial buds

Atypia with buds was seen during carcinogenesis even in the abscence of the ovaries and suggests that the appearance of buds need not be related to the cyclic ovarial function

Slight atypia (grade I) with buds was also observed in some mice receiving acetone without 3 4 benzpyrene This might be due to mechanical stimulation of the cervical area during intravaginal application and need not necessarily indicate carcinogenic potential of the acetone More severe degrees of atypia were not observed in these control animals

Of particular interest was the occurrence



Fig 5 Cervical epithelium of mice after intravaginal application of 3 4 benzpyrene Tip of two ep thel al buds surrounded by a rim of condensed reticulin fibres (× 1100)



Fig 6 Cervical epithelium of mice after intraviginal application of 3 4 benzpyrene A group of cells breaking through the layers of reticulin fibres surrounding the tip of an ep thelial b d (× *1100*)

of buds in apparently normal epithelium after 2 months of application of 3 4 benz pyrene or of acetone alone None of 10 normal untreated control mice and none of 17 mice painted during 1 month with 3,4 benzpyrene or acetone in Table 1 and 2 pre sented epithelial buds These findings suggest that atypias and buds do not develop as a joint phenomenon, bud formation appears to precede cellular atypias

Questionable invasive carcinoma or frank invasive carcinoma were present in some of the mice and the results presented in Table 1 suggest that there is a close association between epithelial buds with atypia and invasive carcinoma Condensation of argentophil reticulin fibres was seen to surround epithelial buds with atypias (Fig 5) Whether this condensation is due to a possible expansion of proliferating buds or represents a reactive process in the stroma could not be determined It should be pointed out that a similar condensation of reticulin fibres was not present in areas with normal epithelium or in those with atypia without buds Minimal penetration of the condensed reticulum at the tip of epithelial buds was observed in four mice (Fig 6) The cells present in the area beyond the reticulum condensation in the stroma were atypical squamous cells with a tendency towards a mature cytoplasm, a phenomenon observed in micro invasive carcinoma of the cervix in human subjects (*Fennell* 1955) Similar reticulin penetration by micro-invasive carcinoma have been found at the tip of buds with carcinoma in situ in the human cervix (*Rubio* & *Lagerlöf* in preparation)

Not all animals developed epithelial atypias with buds Some developed severe atypias but without buds It is to be pointed out however, that invasive carcinoma was always associated with intra epithelial atypias with buds

To sum up our work to date intra epithelial atypia with a smooth epithelial stroma border as well as with epithelial buds projecting into the underlying stroma can be induced in the cervix of mice after protracted application of 3 4 benzpyrene There is apparently a close association between epithelial buds, the degree of cellular atypia and progression to invasive carcinoma Histological evidence suggested that advanced epithelial atypias with buds are severe lesions which may proceed into invasive carcinoma The results are in agreement with earlier results obtained in follow up studies of carcinoma in situ in human subjects (*Rubio* & *Söderberg* 1969 and 1970) In fact, it was observed at follow up that 95 per cent of the 33 patients in whom cervical invasive carcinoma developed had in preceding biopsies carcinoma in situ with epithelial buds Moreover, 95 per cent of 220 cases with micro invasive carcinoma had associated lesions showing carcinoma in situ with epithelial buds

The efficient technical assistance of *M Edenholm* *Y Kock*, *I Kron* and *M Sveander* is gratefully acknowledged

The investigation was supported by a grant from the *Swedish Cancer Society*

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CYTOLOGIC STUDIES IN CASES WITH CARCINOMA *IN SITU* AND MICROINVASIVE CARCINOMA OF THE UTERINE CERVIX

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Cervico vaginal smears from 256 patients with carcinoma in situ and 103 with micro-invasive carcinoma were investigated The malignant cell population was classified according to the predominant cell type into 4 groups, undifferentiated, basal, parabasal, and mature Carcinoma in situ could not be differentiated from micro-invasive carcinoma on the basis of the cytologic appearance in smear preparations, not even by detailed cell analysis including micrometric determinations

It is generally accepted that only after adequate histological study of the cervical area with a large number of sections can the presence of an intraepithelial neoplasia be confirmed and micro invasive carcinoma ruled out Accurate histological diagnosis remains the prime determinant for the treatment of patients with early neoplasias of the cervix (*Margulis et al* 1967)

After study of smear preparations from cases with histological evidence of micro invasive carcinoma, *Fennel* (1954) reported that vaginal smears did not prove to be an indicator of the presence of invasion Recently *Tweedale et al* (1969) found that carcinoma in situ and micro invasive carcinoma could be differentiated on the basis of the cytologic pattern of exfoliated cells Results similar to those obtained by Tweeddale et al were more recently reported by *Ng et al* (1972)

We have analysed cytologic details in malignant cells exfoliated from cases with histological evidence of carcinoma in situ and micro-invasive carcinoma In order to detect possible qualitative and/or quantitative cytological differences between these two lesions, differential cell counts and micrometric determinations were performed

MATERIAL AND METHODS

The basis on which patients were selected for the present study was the availability of representative sections that fulfilled the definition of carcinoma in situ (*Rubio & Soderberg* 1969a) and micro-invasive carcinoma (*Rubio & Soderberg* 1969b) (i e invasion measuring no more than 5 0 mm in maximal size), as well as vagino-cervical smears [illegible]

[illegible] 103 cases of micro-invasive carcinoma that fulfilled these requirements The diagnosis was made on biopsies and/or cone specimens in all 256 carcinoma in situ cases and in 75 of the 103 cases with micro-invasive carcinoma In the remaining 28 cases the diagnosis of micro-invasion was done on one or multiple wedge biopsies

The method utilized for obtaining exfoliated cells varied chronologically as follows until 1961, exfoliated cells were obtained by aspiration of the

Received 6 ix 73 Accepted 26 ix 73

vaginal pool according to the method advocated by Papanicolaou Thereafter the cells were obtained by rubbing the cervical canal with a cottonswab applicator, as recommended by Day, and the portio vaginalis either with a cotton applicator or by means of an Ayre's spatula

Cytologic Studies

The malignant cell populations in the material were classified as reported previously (*Rubio & Söderberg* 1966 and 1968) on the basis of the morphological appearance of the predominant cell type into four groups (undifferentiated basal parabasal and mature cell types) Predominance means that at least 50 per cent of the malignant cells could be classified as one of the four cell types

a) *Microscopic Description of the 4 Cell Types*

Undifferentiated type (Fig 1) The cells in this group did not resemble any of the normal cells of the squamous epithelium of the cervix The nuclei were rounded with well defined irregular nuclear membrane The chromatin was arranged in small granular clumps or as a thin network of chromatin with a few irregular chromocentres The cytoplasm was scanty, poorly stained and sometimes

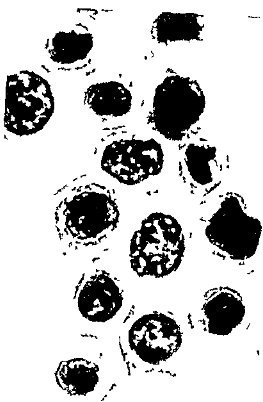


Fig 2 Basal cell type × 1400



Fig 1 Undifferentiated cell type × 1400

apparently absent, if present, the nuclear-cytoplasmic ratio was large The cells generally appeared to form syncytical clusters

Basal cell type (Fig 2) Resembled normal basal squamous cells The cells were rounded with a large nucleus The chromatin was coarsely clumped and angulated The clumps were usually connected by irregular, thick threads of chromatin The nuclear membrane was thick and the cytoplasm was scanty usually cyanophilic with well-defined cell borders The nuclear cytoplasmic ratio was large The cells were seen as single cells or in rows (probably due to an smearing artefact, since they were often seen within stripes of mucus)

Parabasal cell type (Fig 3) The cells were larger than the preceding cell type The nucleus was oval with granular clumps of chromatin connected by thin threads The cytoplasm was usually eosinophilic with sharp cell borders The nuclear-cytoplasmic ratio was smaller than in the basal *cell* type These cells were seen either singly or in clusters

Mature cell type (Fig 4) Characterized by the presence of spindleshaped cells The nucleus was elongated with large angulated chromatin clumps giving the nucleus a pyknotic appearance at low power The cytoplasm was relatively large and



Fig 3 Parabasal cell type × 1400



Fig 4 Mature cell type × 1400

stained eosinophil c or cyanophil c The cells oc curred in large sheets with indistinct cell borders sometimes in syncytical formations In single cells the nuclear cytoplasmic ratio was larger in the transverse diameter of the cell than along the ma jor axis

b) *Differential Cell Counts*

All malignant cells occurring in smears from 37 cases of carcinoma in situ were recorded The ma lignant cells were classified as mentioned above in undifferentiated, basal parabasal and mature cell types Fourteen consecutive cases were chosen from the group of 87 cases in which basal cell types predominated 17 from the group of 121 cases in which parabasal cell types predominated 3 from the group of 15 cases presenting mature cell types and 3 from the group of 12 cases in which undiffe rentiated cell types predominated

c) *Micrometric Studies*

Micrometric studies of 1 542 malignant cells were performed in 10 cases of carcinoma in situ and of 602 malignant cells in 6 cases of micro-in vasive carcinoma Micrometric determinations were also performed in 1000 normal parabasal cells from 10 healthy women without gynaecological symptoms and with normal vaginal smears Both mal gnant and normal cells were chosen after screening the smear preparations as recommended by Koss *(Koss & Durfee* 1961)

The largest cell diameter and the largest nuclear diameter were measured by means of a Zeiss cali brated ocular micrometer Measurements were per formed under oil immersion (× 1250) Based on the 2 measurements the nuclear cytoplasmic ratio was calculated

Micrometric determinations were done only in cases in which basal and parabasal malignant cell types were predominant Similar microdetermina tions on mature and undifferentiated cell types were not done since the latter cell types usually exhibited a tendency to gather in cohesive clusters with ill defined cellular borders often giving rise to apparent syncytial sheets These features made cell measurements on mature and undifferentiated cell types difficult and unreliable

TABLE 1 *Predominant Cell Types on Smear Preparations from 359 Women 256 with Carcinoma in situ and 103 with Micro invasive Carcinoma*

Cell types	Carcinoma in situ		Micro-invasive carcinoma	
	No cases	Per cent	No cases	Per cent
Basal	87	34	34	34
Parabasal	121	47	46	45
Mature	15	6	7	7
Undiffer	12	5	8	7
Mixed	21	8	8	7
Total	256	100	103	100

RESULTS

A) *Classification of Smear Preparations According to the Predominance of Cell Types*

There was a predominant cell type in 92 per cent of the cases of carcinoma in situ and in 93 per cent of those of micro-invasive carcinoma. The results presented in Table 1 show that there was no apparent difference in the proportion of predominant cell types in cases of carcinoma in situ and micro invasive carcinoma

B) *Differential Cell Counts on Malignant Cell Populations*

The results of this study are shown in Table 2 It is seen that, in cases in which undifferentiated cell types visually are considered to predominate, 76 per cent (mean) of the total malignant cell population were by differential cell counts recorded as undifferentiated cells The corresponding figures obtained in cases in which basal cell types predominated was 83 per cent (mean) The rates applying to cases with parabasal and mature cell types were 69 per cent and 79 per cent, respectively (Cfr Table 2)

In 36 of the 37 cases the predominance of one of the four cell types investigated varied from 54 per cent to 95 per cent Only in one of the 37 cases studied (i e 3 per cent) in which all malignant cells were counted, the predominance of a particular cell type was less than 50 per cent In this case, on primary visual impression considered to have predominantly parabasal cell types counts showed only 46 per cent of malignant parabasal cells The remaining 54 per cent of the malignant cells were classified as follows 33 per cent basal type, 13 per cent mature, and 8 per cent undifferentiated

C) *Micrometric Stud es*

a) Cytoplasmic size The results of cytoplasmic measurements are depicted in Fig 5 Cell measurements on 1,069 malignant cells

TABLE 2 *The Frequency of Predominant Cell Types in the Total Ma' Cell Population in 37 Cases of Carcinoma in situ of the Cervix*

	cell type/total number			t cells
	Undifferent., (3 cases	Basal -s)	(17	Mature -s)
Per cent of total (mean)	1 614/2 1 76 per cent	3 •	19 809/ 69 per	+16
Range	71–86 per cent		46–95	

Total numbe:

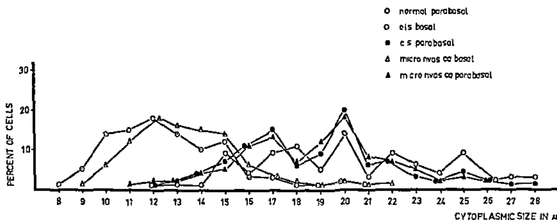


Fig 5 The cytoplasmic size in 2 144 malignant cells of cases with carcinoma in situ and micro-invasive carcinoma in which basal and parabasal cell types predominated Measurements done in 1 000 parabasal normal cells are also shown

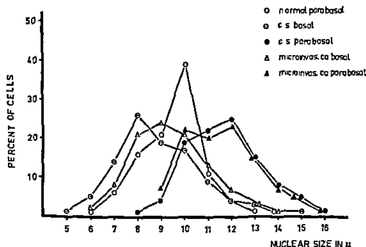


Fig 6 The nuclear size in 2 144 malignant cells from cases of carcinoma in situ and micro invasive carcinoma in which basal and parabasal cell types predominated Measurements done in 1 000 parabasal normal cells are also shown

in smears with predominantly basal cell types demonstrated that the majority of the cells measured 15 micrometers or less This was true both in cases of carcinoma in situ and micro invasive carcinoma Among the 768 cells measured in carcinoma in situ cases, 84 per cent measured 15 micrometers or less In micro-invasive carcinoma the rate was 82 per cent in the 301 cells measured

Cell measurements were also done on 1,075 malignant cells in smears presenting predominantly parabasal cell types. The results demonstrated that the majority or the cells measured 15 micrometers or more Among the 774 cells measured in cases of carcinoma in situ, 93 per cent measured 15 micrometers or more while the rate in 301 cells from cases of micro-invasive carcinoma was 92 per cent These results indicate no essential differences in the cytoplasmic size in apparently similar cell types in carcinoma in situ and in micro invasive carcinoma cases

TABLE 1 *Predominant Cell Types on Smear Preparations from 359 Women, 256 with Carcinoma in situ and 103 with Micro invasive Carcinoma*

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C) *Micrometric Stud es*

a) Cytoplasmic size The results of cytoplasmic measurements are depicted in Fig 5 Cell measurements on 1,069 malignant cells

TABLE 2 *The Frequency of Predominant Cell Types in the Total Malignant Cell Population in 37 Cases of Carcinoma in situ of the Cervix*

	Predominant cell type/total number of malignant cells			
	Undifferentiated (3 cases)	Basal (14 cases)	Parabasal (17 cases)	Mature (3 cases)
Per cent of total (mean)	1 614/2 132 76 per cent	10 326/12 408 83 per cent	19 809/28 833 69 per cent	2 694/3 416 79 per cent
Range	71–86 per cent	62–95 per cent	46–95 per cent	76 89 per cent

Total number of cells investigated in the 37 cases 46 789 (1 268 cells per slide, range 205–6 629 cel s)

invasive carcinoma is treated by radiotherapy or hysterectomy, the differential diagnosis between these two lesions before conization remains a challenge to the cytopathologist In fact, complete healing of cervical tissue is required before radiotherapy can be instituted This means that treatment of patients with micro-invasive carcinoma not proven until at conization may be delayed by several weeks

We wanted therefore to analyse the possibility of differentiating carcinoma in situ from micro-invasive carcinoma on smear preparations containing fair amounts of malignant cells The method by which cells were collected could prove to be of importance In two other reports on this subject (*Tweeddale et al* 1969 and *Ng et al* 1972), two different methods of cell collection have been used In addition to cervical scraping *Tweeddale et al* (1969) used endocervical swab while *Ng et al* (1972) used pipette aspiration from the cervical canal The method by which cells are collected in the present survey was not the same for the whole material (cf material and methods) The argument that it is impossible to differentiate carcinoma in situ from micro invasive carcinoma on smear preparations however, was valid if pipettes were used for the collection (i e before 1961) and in cases in which the material was obtained by endocervical swab and cervical scraping (i e after 1961)

It would appear that the method of collection is of no fundamental importance in cell analysis if representative amounts of well preserved malignant cells are present in the preparations

In the present material—as in previous reports (*Rubio* & *Soderberg* 1966 *Rubio* & *Soderberg* 1968 and *Rubio* 1971)—the malignant cell population was classified according to the predominant cell type in smears Other authors have provided similar classification according to dominant cell types *Boddington et al* (1960) and *Timonen* & *Kauranemi* (1967) recognized 6 predominant malignant cell types and *Tweeddale et al* (1969) 5 types The primary subjective impression obtained by us and used as a basis on which to classify smear preparations according to the predominant cell type was corroborated by the detailed analysis of malignant cells in differential cell counts By this method the predominant cell type in 36 of the 37 cases investigated was confirmed It is thus apparent that the primary subjective impression represents a reliable basis on which to classify smear preparations according to the predominant cell type On this background the malignant cell population on smears from cases with micro-invasive carcinoma was analysed The evidence indicates that there is no essential difference in the frequency of predominant cell type in micro-invasive carcinoma and in carcinoma in situ

Tweeddale et al found that the smears from cases with micro invasive carcinoma commonly contained non syncytial clusters In contrast *Ng et al* found that the characteristic of micro-invasion appeared to be the syncytial arrangement of the cells In the present study, the frequency of cell types more prone to form syncytial sheets (mature and undifferentiated) was similar in carcinoma in situ and in micro-invasive carcinoma

Reagan et al reported differences in nuclear measurements in cells from carcinoma in situ (*Reagan* & *Patten* 1961) and from micro invasive carcinoma (*Ng et al* 1972) *Tweeddale et al* however, found no significant differences in nuclear measurements in the cells from these two lesions Like *Tweeddale et al* we found no appreciable differences in nuclear measurements in carcinoma in situ and in micro invasive carcinoma

To sum up the present survey carcinoma in situ and micro invasive carcinoma could not be differentiated by the cytologic pattern in smears even after application of a detailed cell analysis including micrometric determinations It may be of interest to mention that in a recent study of the non neoplastic components of the vaginal cervical smears from patients with carcinoma in situ and micro-invasive carcinoma no essential differences could be recorded (*Rubio* 1973)

I am indebted to professor *B Lagerlöf* and dr *I Zajicek* for interest in and constructive criticism of this investigation

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Acta path microbiol scand Section A 82, 169–174, 1974

LOCALISATION OF HUMAN α-FOETOPROTEIN SYNTHESIS IN HEPATOBLASTOMA CELLS BY IMMUNOFLUORESCENCE AND IMMUNOPEROXIDASE METHODS

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By means of indirect immunofluorescence (IF) and indirect immunoperoxidase (IP) technique the presence of α foetoprotein (AFP) has been demonstrated in foetal liver tissue as well as in liver tissue from two cases of hepatoblastoma In both cases of hepatoblastoma the serum AFP were determined by rocket immunoelectrophoresis Removal of the tumours caused a rapid fall in the concentration of serum AFP

Hepatoblastoma is a distinctive tumour occuring almost exclusively in the early childhood The microscopic appearance is characterized by a resemblance to embryonal and foetal liver

The tumour has a well defined histological picture which can be differentiated from other hepatic tumours (18)

α_1-foetoprotein (AFP) was first demonstrated in serum from the human foetus by *Bergstrand & Czar* in 1956 (4), and this finding has later been confirmed by a number of authors (for review see *Abelev* (1)) Synthesis has been shown to take place in the foetal liver and yolk sac (7, 9) In mature infants the concentration of AFP in serum is very low a few weeks after birth (12), and in normal infants and adults it can only be measured by a sensitive radio-immunoassay (RIA) as described by *Ruoslahti & Seppälä* (14) Reappearance in blood of AFP in higher concentrations is seen in various non-tumour liver disorders especially during the first year of life (1), as well as in hepatoblastoma (1 2), in hepatocarcinoma (1, 13, 20), in pregnancy (19), and infrequently in teratoblastoma of the ovaries and testes (6)

Recent investigations have demonstrated the presence of AFP in primary adult liver cancer (hepatoma) tissues by means of a immunofluorescence technique (IF) (5, 8, 10, 13)

In one case of hepatoblastoma in a 12 months old boy, Alpert and Seeler has found a high serum concentration of AFP (2) However, attempts to localize AFP in hepatoblastoma cells have not yet been carried out This study presents two cases of hepatoblastoma with a high concentration of AFP both in serum and in tumour cells The indirect IF

Received 26 vii 73 Accepted 12 ix 73

technique and the indirect immunoperoxidase (IP) technique have been used for localization of AFP in the hepatoblastoma tissue

MATERIAL AND METHODS

Case No 1

A boy aged 2, was admitted for investigation of *anaemia and enlargement of the liver* At laparotomy the right liver lobe, containing a large tumour, was resected Histologically the tumour was a hepatoblastoma of purely epithelial origin Following surgery the patient was given Actino mycin D and later Endoxan® The boy remained reasonably well for several months, but was later readmitted with a tumour in the epigastric region He died one year after the initial admission At post mortem, the liver was largely replaced by confluent masses of white tumour tissue Wide spread metastases from hepatoblastoma were found in the lungs

Case No 2

A 3 year old Greenlandic boy was admitted for investigation of an abdominal mass Two years previously he had been treated for molluscum contagiosum in the face At laparotomy a large tumour, involving the right liver lobe, was removed

Histological examination showed the tumour to be a hepatoblastoma of purely epithelial origin The patient died of pulmonary oedema ten days after the operation At autopsy there was no macro scopic or microscopic evidence of remaining tumour tissue

Antigens

Serum antigens Before laparotomy and during the first weeks after removal of the tumours, sera were collected for quantitation of AFP Furthermore, cord blood sera were collected from cases of induced abortions of a gestational age between 16 and 20 weeks

Tissue antigens Liver tumour tissue was obtained during operation from the two cases of hepatoblastomas Furthermore, foetal liver tissue was obtained from several abortions, and normal liver tissue was obtained from a healthy patient

The tissue samples were cut into small blocks and half of the samples were fixed in cold 95 per cent ethanol and half were frozen with dry ice

Sections of tissue paraffin embedded according to *Sainte Marie* (17) as well as sections of frozen tissue were used for the study of AFP

Antisera

1 Specific AFP antiserum (rabbit immunoglobulins (IgA + IgG) against human α_1-foeto protein, Dakopatts, Copenhagen, Denmark) has been used No reaction with other foetoproteins or adult proteins was observed if tested by crossed immunoelectrophoresis

2 Fluorescein isothiocyanate (FITC) conjugate (Fluorescein conjugated swine against rabbit serum IgG, Dakopatts, Copenhagen, Denmark) has an average conjugation degree of approximately 2 3 moles FITC per mole of protein as calculated from the optical density ratio at 495/280 nm

The mean optical density ratio 495/280 nm of the conjugated were 0 66 with 0 30 as lowest and 0 95 as highest ratio Unconjugated immunoglobulins with optical density ratio above 0 95 were removed by ion exchange chromatography The antibody titre of the conjugates was 100 i e 1 ml conjugate absorbs 100 micrograms pure human immunoglobulin By chess board titration (21) a working titre of 1 20 was obtained

3 Peroxidase conjugate (Peroxidase conjugated swine against rabbit serum IgG, Dakopatts, Copen hagen, Denmark) of a molar peroxidase/protein ratio of 1 20 was used A titre of 1 20 for th s conjugate was obtained by chess board titration (21)

Staining Procedure

Both the IF staining and the IP staining were performed as a double layer technique (3, 5, 10 13) Sections were deparaffinized and washed for three periods of 5 minutes in phosphate buffered saline (PBS, pH = 7 2) Cryostat cut sections were air dried for 15 minutes As the first layer, in cubation at room temperature in a moist chamber with *diluted AFP antiserum (1 10) was carried* out for 30 minutes, followed by three washings for 5 minutes in PBS at pH = 7 2 Finally incubation with the conjugate was carried out for 30 minutes followed by three washings as above The described procedure was identical for both the IF and the IP technique

IF sections were mounted in PBS pH = 7 2 with 10 per cent glycerol and examined with a Leitz Ortoplan® fluorescence microscope modified with a Tryoda® wide angle darkfield oil immersion condenser The light source was an Osram *HBO 200* lamp and the primary filter was a *FITC inter ference* filter with red contrast band (15 16) (Laboratory for Technical Optics Lyngby, *Den* mark) The secondary filter was a 2 mm glass filter (Schott & Gen Mainz Germany) matched *to fit* the primary filter (15 16)

IP sections were placed in a substrate solution for visualizing the enzyme (3 *Incubation* was *carried* out for 30 minutes followed by several washings in PBS Haematoxylin-eosin staining was carried out on one of the serial *sections for* histological control diagnosis

TABLE 1 *Controls for Establishing Specificity of Immunoperoxidase and Immunofluorescence Staining*

Antigen	Antiserum	Conjugate	Results of staining reaction
Foetal liver or Tumour tissue	Rabbit anti α foetopro ein serum	Labelled antirabbit immunoglobulin	Positive
Foetal liver or Tumour tissue	*Saline*	*Labelled antirabbit* immunoglobulin	*Negative*
Foetal liver Tumour tissue	Absorbed rabbit α foeto protein serum or non immune rabbit serum	Labelled antirabbit immunoglobulin	
*Foetal liver or Tumour tissue	Rabbit anti α foetoprotein serum	Unlabelled antirabbit immunoglobulin—followed by peroxidase reaction	Negative
Normal liver	Rabbit anti α foetoprotein serum	Labelled antirabbit immunoglobulin	Negative

* For peroxidase reactions only

Controls

The specificity of the staining reaction was investigated as shown in Table 1

Normal liver tissue was treated in the same manner as the tumour and foetal liver tissue Tumour and foetal liver tissue control sections were treated first with non immune rabbit serum instead of AFP antiserum or with AFP antiserum absorbed with a slight excess of pure AFP AFP was purified from foetal serum as described by *Nørgaard Pedersen* (11)

Quantitation of Serum AFP

Serum AFP was determined by rocket immunoelectrophoresis (11) Antigen α_1 foetoprotein standard 0 5 ml 220 mg/l (Behringwerke AG Marburg)

Antibody rabbit immunoglobulins to human α_1 foetoprotein (Dakopatts, Copenhagen)

RESULTS

Serum Concentration of AFP

In case no 1 the concentration was 308 mg/l before operation 4 weeks later the concentration was less than 1 0 mg/l and after six months the concentration had raised to 468 mg/l In case no 2 the initial serum concentration was 206 mg/l and 10 days after the operation only 36 mg/l The AFP concentrations in the foetal sera were all above 1000 mg/l

Localization of AFP

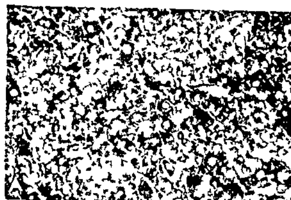
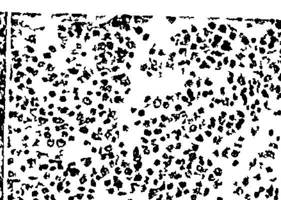
The IF and IP technique showed the same staining result

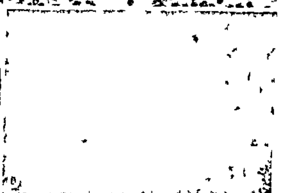
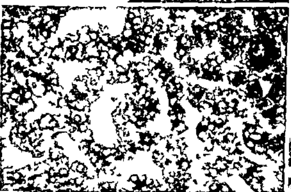
A positive reaction was seen as a granular staining of the cytoplasm of the cells (Fig 1) In the foetal liver the majority of hepatocytes reacted positively, but negatively reacting cells were always found as well The positive reaction varied in intensity, some cells showed a weak reaction while others reacted strongly positive (Fig 1 A and C) In the tumour tissue staining reactions were similar to those of foetal tissue, although the positive reaction was generally of lower intensity (Fig 1 E and G) Only tissue samples fixed in cold 95 per cent ethanol were found suitable for the described staining techniques With the corresponding cryostat sections a rather heavy unspecific staining were observed

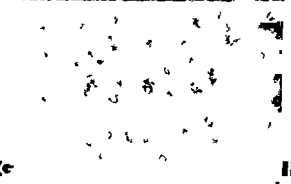
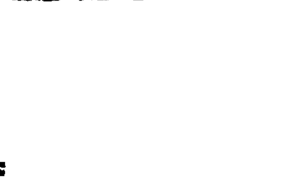
In normal liver tissue no staining was found whether the IF or the IP technique were used

DISCUSSION

By means of indirect IF and IP techniques and with specific purified AFP antiserum, we have demonstrated a positive staining reaction both in foetal liver cells and in two cases of hepatoblastoma of pure epithelial type

Both techniques were suitable, but since a stable staining product is only seen if the IP technique is used, this type of staining reaction is preferred Furthermore, the peroxidase staining will permit ultrastructural localization of the AFP production site

In hepatoblastoma cells the epithelial cellular localization of AFP was seen as a granular staining of the cytoplasma A special fluorescent line of the perinuclear zone and the cytoplasmic membranes, as found by *Nishioka et al* (10) in hepatocarcinoma cells, was not seen in hepatoblastoma cells

Only the paraffin sections of tissues fixed in 95 per cent cold ethanol were suitable for the IF and IP staining A rather heavy unspecific staining was seen in the cryostat sections The same finding has been reported by *Engelhardt et al* (5)

The localization of AFP in the tumour cells, the fall in serum AFP concentration after surgical removal of the tumour, and finally the increased concentration of AFP in the serum in reappearance of tumour tissue clearly indicate that the site of AFP production is in hepatoblastoma tumour cells The higher level of AFP in foetal sera and the more intensive staining of the foetal cells compared with tumour cells indicate relationship between serum AFP concentration and staining intensity in the corresponding cells Even in normal, healthy individuals it is possible by RIA to measure very small amounts of AFP in the serum (14) Consequently, the serum quantitation seems to be more sensitive and faster than the more laborious histological staining reactions

Fig 1

A IP staining for AFP in foetal liver tissue Positive s aining cells are seen as cells with brown cytoplasma × 250

B HE sta ning in neighbouring section × 250

C Positive IF staining of foetal liver tissue Cells with bright green fluorescence in the cyto plasma are positive reacting cells × 60

D Negative control of the IP staining Absorbed rabbit α_1 foetoprotein antiserum has been used × 250

E IP staining for AFP in the hepatoblastoma tissue Cells with brown cytoplasma are positive reacting cells × 250

F HE staining in neig bouring section × 250 250

G Positive IF staini g of the hepatoblastoma tissue Cells with b ght green fluorescence in the cytoplasma are [s ve reacting cells × 250

H Negative contro [he IP s aining of the hepatoblastoma tiss Absorbed rabbit α_1 foetop o tein antiserum l as been used × 250

Dr *H Andersen* head of the Laboratory of Cyto and Histochemistry Anatomy Department A University of Copenhagen, Prof *J Falck Hansen*, Department of Gynaecology, Bispebjerg Hospital and Prof *G Stakemann*, Department of Gynaecology and Obstetric, Øresundshospitalet have kindly provided us with foe al tissue and sera

Thanks are due to Prof *T Schiødt* University Institute of Pathological Anatomy and Dr *Inge Tygstrup*, head of the Laboratory of Paediatric Pathology, for the classification of the tumours Dr *K Mauritzen* head of the Department of Paediatric Surgery are gratefully acknowledged for supplying us with tumour tiss e

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Acta path microbiol scand Section A 82, 175–178 1974

THE EFFECT OF TEMPERATURE ON INITIAL CELL ADHESION

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The effect of temperature on the adhesion of cells to glass was studied The initial cell adhesion was found to be temperature dependent within the range 4°–37° C The initial cell adhesion was independent of temperature changes after formaldehyde fixation of the cells or after arresting cell metabolism with 2 4 dinitrophenol and iodoacetate It is suggested that cell surface functions dependent on cellular metabolism facilitates initial cell adhesion

Changes in temperature has been reported to influence the adhesiveness of cells both in cell to cell adhesion (*Zeidman* 1949, *Orr* & *Roseman* 1969) and in cell adhesion to glass (*Moscona* 1961, *Weiss* 1964)

Cell adhesion to glass has been suggested to occur by two stages (*Taylor* 1961), an initial adhesion occurring upon contact, and a secondary stronger interaction which has been associated with flattening of the cell, synthesis of cell surface materials and development of cellular structures such as hemidesmosomes This kind of adhesion has by some authors been termed 'attachment' It has been suggested that the bonding mechanisms may be different in initial and secondary adhesion (*Takeichi* 1971) but others fail to recognize such differences

Much of the information on the temperature effect on cell adhesion has been obtained by methods which do not differentiate between initial and secondary adhesion The aim of the present study was to evaluate the effect of temperature on initial adhesion of cells to glass For measuring the initial cell adhesion, a system with eluting cell suspensions through glass bead columns at predetermined flow rates was used (*Attramadal* & *Jonsen* 1970)

Received 22 ix 73 Accepted 22 ix 73

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MATERIALS AND METHODS

Cells P 388 cells (mouse lymphoblastoma cells) were propagated as suspension culture in Eagle's minimum essential medium (MEM) (Grand Island Biological Company, New York USA) Per litre medium was added 1 2 g methocel (methocel MC 4000cP Fluka A G, Switzerland), 1 g pluronic acid (Marles Kuhlmann Wyandotte, France), 10 000 I U penicillin and 10 mg streptomycin (Glaxo Laboratories, Greenford England) and 100 ml foetal bovine serum (Grand Island Biological Company, New York, U S A)

Glass bead columns were made as previously described (*Attramadal* & *Jonsen* 1970) Five grams of glass beads 0 5 mm in diameter were filled in PVC plastic tubing of 5 mm diameter

Evaluation of initial cell adhesion Cells spun down from stock culture were suspended in MEM with 5 per cent foetal calf serum to a concentration of 4 10^4 cells/ml After equilibration to the desired temperature 1 ml of cell suspension was applied to a glass bead column at a rate of 2 5 ml/minute Elution of the cells followed immediately using MEM at the same flow rate The first 3 ml

of the eluate were collected for counting, and cells adhering to the glass beads were calculated as the difference between the total number of cells applied to the column and the number of cells recovered in the eluate

The cell number was counted using an electronic particle counter (Celloscope, AB Lars Ljungberg & Co, Stockholm, Sweden) Cell suspensions diluted in 0 15 M NaCl were aspirated through a capillary with a 70 nm diameter

Formaldehyde fixation of cells was performed by suspending cells in a 2 per cent solution of reagent grade formaldehyde in MEM for 1 hour at 22° C The cells were then centrifuged and resuspended in Eagle s MEM with 5 per cent foetal calf serum

ATP determination Cell suspensions to be assayed for ATP were centrifuged and suspended in MEM at a concentration of 2 10^6 cells/ml 3 ml of this suspension was boiled for 10 minutes in a water bath After centrifugation, 0 2 ml of the supernatant was diluted with 1 6 ml Tris buffer, 0 015 M, pH 7 4 in a counting vial 0 2 ml firefly lantern extract in 0 05 M potassium arsenate and 0 02 M $MgSO_4$ at pH 7 4 (Sigma Chemical Company, U S A) was then added the vials were placed in a Tricarb liquid scintillation counter, model 3365 and the luminescence was determined in the ^3H channel The counting time was 6 seconds, and the counting was repeated 5 times in succession by switching the Tricarb to ' repeat counting The counts were extrapolated to zero time on a graph ATP standards were made with samples containing 10^{-11}–10^{-10} mol ATP (Sigma Chemical Company U S A)

RESULTS

At 22° C about 25 per cent of the live cells adhered to the glass bead columns At 4° C the adhesion rate was reduced to about 12 per cent, and at 37° C the adhesion rate was about 30 per cent The initial adhesion of the cells thus showed a significant temperature dependence (Table 1)

TABLE 1 *The Initial Adhesion of P 388 Cells at Different Temperatures*

Temperature (C)	Per cent adhering cells	Standard error	Number of observations
4	11 6	2 3	22
22	24 0	2 6	25
37	31 1	5 2	23

TABLE 2 *The Initial Adhesion of Formaldehyde fixed Cells at Different Temperatures*

Temperature (C)	Per cent adhering cells	Standard error	Number of observations
4	11 7	2 4	15
22	13 6	2 9	25
37	12 0	2 9	17

TABLE 3 *The Initial Adhesion of Dinitrophenol and Iodoacetate Treated Cells at Different Temperatures*

Temperature (C)	Per cent adhering cells	Standard error	Number of observations
4	22 3	3 9	18
22	19 0	2 6	17
37	21 7	3 8	18

Within the same temperature range the adhesion rate was independent of temperature when formaldehyde fixed cells were used Such cells had also a lower adhesion rate than the control cells (Table 2)

The cellular ATP was reduced to 2 75 p *moles* ATP/10^5 cells after treating the cell cultures with 1 mM 2,4 dinitrophenol and 2 mM iodoacetate for 20 minutes as compared to 0 3 n moles ATP/10^5 cells in untreated cultures Cells treated with dinitrophenol and iodoacetate still adhered to the glass beads, but no temperature dependence was observed (Table 3)

The elution pattern of the non adhering cells from glass bead columns was recorded for dinitrophenol and iodoacetate treated cells, and for untreated control cells Fig 1 shows the distribution pattern from 1 ml samples containing 4 10^6 cells No significant difference in the "trailing of the cells was detected

DISCUSSION

Initial cell adhesion has mainly been investigated by detaching cells from the substratum after adhesion has occurred and lasted for

some time The glass bead column technique employed in the present study records the cell adhesivity immediately upon contact with the substratum, and is thus thought to distinguish the initial cell adhesion from subsequent adhesion phenomena The technique is based on the elution of a cell suspension through a glass bead column at a predetermined flow rate (*Attramadal & Jonsen* 1970) The flow rate is so adjusted that the kinetic energy of the streaming cells is of the same magnitude as the adhesion force between cells and the glass surface upon contact Cells which collide with the glass bead surface will either adhere, or be moved further on if the kinetic energy of the streaming fluid is stronger than the initial adhesion forces The reduction of cells in the cell suspension after passage through a glass bead column is thus a measure of the initial adhesive force

The elution pattern of cell aliquots from glass bead columns were similar for cells with

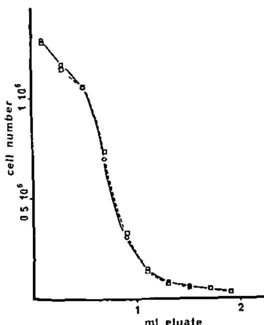


Fig 1 The distribution of non adhering cells in the eluate from glass bead columns after elution of 1 ml suspension of dinitrophenol and iodoacetate treated cells continuous line) and untreated control cells (broken line)

arrested metabolism and control cells (Figure 1) If metabolizing cells develop secondary adhesion to the glass beads as the cell suspension passes through the column this would be revealed by a different elution pattern compared to non metabolizing cells Initial adhesion only would result in a larger fraction of cells to detach after contact was established and this would cause trailing of the cells in the eluate pattern The results thus indicate that no secondary adhesion mechanisms were involved in the test system

The results in Table 1 demonstrate that the initial adhesion of P 388 cells to glass is temperature dependent An increase in the adhesion rate when the temperature is increased could either be a result of physico chemical mechanisms in the medium or in the cell surface One mechanism could be the drainage effect which occurs when two surfaces are approaching and the intervening medium is forced out of the gap between the surfaces as discussed by *Curtis* (1962) However the temperature independent adhesion of formaldehyde fixed and metabolism arrested cells (Tables 2 and 3) indicates that the initial cell adhesion is not primarily associated with temperature dependent physical properties of the medium Nor is cellular metabolism a prerequisite for the initiation of cell adhesion It appears that initial adhesion is principally mediated by physico chemical interactions between components on the cell surface and the glass surface and that this adhesion is modified by cell functions which are dependent on cell metabolism The present findings do not suggest the nature of such temperature dependent cell adhesion mechanisms However, cell membrane movements and the formation of pseudopods are functions brought about by metabolic activity which according to *Weiss* (1964) and *Bangham & Pethica* (1960) greatly enhance the initiation of cell adhesion Moreover the resistance of the cell to surface deformations is lower at higher temperature (*Mitchison & Swann* 1954)

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Acta path microbiol scand Section A 82, 179 181, 1974

GASTRIC LOCALIZATION OF ENDODERMAL SINUS TUMOUR

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The rare, malignant germ cell tumour, endodermal sinus tumour, may also have an extra gonadal localization A case of gastric localization is presented Palliative resection of the bleeding tumour was performed including partial hepatectomy A cavity developed between the abdomen and the left half of the thorax, which became infected postoperatively, resulting in septicaemia and death in the course of renewed attempts at drainage At autopsy an adult teratoma was found in the one testis, just as teratomata had been removed three years previously from both lung hila It is suggested that the endodermal sinus tumour in the stomach may have been a metastasis from one of these teratomata or a stage in a multiple teratoid anlage

Endodermal sinus tumour is a histologically well defined germ cell tumour form, described for the first time with gonadal localization by *Teilum* (1959, 1965)

The tumour is characterized by being composed of the extra embryonic endoderm and mesoblast in specific structures, which are decisive for the identification of this neoplasm Endodermal sinus tumour has also been described in extra gonadal localizations in the anterior mediastinum (*Teilmann et al* 1967), in the pineal gland (*Bestle* 1968, *Albrechtsen et al* 1972), in the sacro-coccygeal region (*Rao et al* 1964) and in the vagina (*Vawter* 1965 *Kraus* 1967)

The present report describes a case of this rare tumour localized to the fundus of the stomach in a 23 year-old man with adult teratomata in the testis and mediastinum

Received 3 ı 73 Accepted 30 ix 73

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CASE REPORT

A 23 year old man was given blood transfusions following melaena and transferred to Rigshospitalet after a roentgenogram of the stomach had shown a tumour localized to the fundus

At the age of 19 years he had had fever and generalized lymphadenitis, and a roentgenological examination showed lumpy masses in both lung hila Thoracotomy was performed in two sittings, with removal of a tumour 6 cm in diameter and then a tumour 3 cm in diameter, both lying around the main bronchi (teratomata)

Physical signs on admission His general condition and nutritional state were good The abdomen was soft without tenderness or masses, no hepatomegaly or splenomegaly Genitalia externa and rectal exploration showed nothing abnormal

Investigations On admission haemoglobin was 6 9 mmol/l (reference 7 9–10 5), clotting time 7½ min (reference 5–12), bleeding time 2¾ min (reference <5), ESR 32 mm/h (reference <6), serum creatinine 0 10 mmol/l (reference 0 06–0 13), serum bilirubin 8 µmol/l (reference 2–17) phosphatase 23 U/l (reference 13 38), alanine-amino transferase 9 U/l (reference 5–25) A roentgenogram of the oesophagus was normal, but the stomach showed a large space-occupying broad based tumour in the fundus The remainder of the stomach bulbus duodeni and duodenal arch were

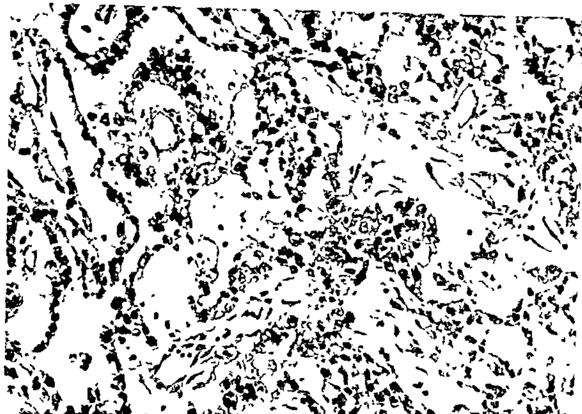


Fig 1 Photomicrograph of a gastric endodermal sinus tumour causing severe gastrointestinal bleeding in a man aged 23, leading to palliative resection and partial hepatectomy

normal Gastroscopy showed an ulcer on the greater curvature at the transition to the fundus, approximately 1½ cm in size, with a raised edge, greyish-white base and patchy bleeding A biopsy could not be obtained

Treatment Left sided thoracotomy exposed a tumour the size of two fists, localized to the fundus of the stomach and penetrating all the layers of the stomach into the left lobe of the liver Numerous enlarged lymph nodes were felt along the greater curvature of the stomach, and frozen section microscopy showed carcinoma The tumour was considered resectable, and in view of the severe haemorrhages experienced by the patient palliative intervention was performed The left liver lobe was resected close to the inferior vena cava leaving the denuded liver surface covered with resorbable gelatine (Spongostan®) The tumour was then removed along with part of the diaphragm, and a 2 layered oesophagogastrostomy was performed

Course Roentgenograms of the thorax showed a massive opacity over the whole left half of the thorax, disappearance of the left diaphragmatic arch and considerable enlargement of the heart, which together with the mediastinum and trachea was displaced to the right, resulting in partial compression of the right lung Artificial ventilation was given and the patient was tracheostomized Penicillin and streptomycin were administered but the temperature fluctuated strongly throughout the 24 hours *Citrobacter freundii Streptococcus faecalis* and *Proteus Morgani* could be grown from all thoracic drains and from the abdominal drain and also a few *Escherichia coli* from the latter Secretion from the drains became bilestained, and it was assumed that a cavity abutted the denuded liver surface and extended through the diaphragm to the left half of the thorax Tests showed impaired liver function The remaining thorax drain fell out and the patient developed a septicaemic fever (*E coli)* Drainage was again necessary, and was done via the previous drainage site but massive venous bleeding with intractable shock occurred and death ensued

Surgical Pathology

The operation specimen consisted of 3 cm of oesophagus the upper half of the stomach the omentum the spleen and the left lobe of the liver The incised ventricle showed a 5 × 8 cm large ulcerating tumour on the posterior wall of the fundus growing into the adherent left liver lobe The tumour tissue was greyish and necrotic, and

extended laterally under the mucosa covered wall of the stomach The spleen was normal without any tumour infiltration A few large lymph nodes along the greater curvature were infiltrated with tumour The histological picture was characteris ic of endo dermal sinus tumour with (1) a loosely vacuo lated network with widely meshed cystic spaces, lined with flat mesotheloid cells, PAS positive glo bules were seen in the meshes (2) a myxoid reti culin resembling the 'magma reticulare' or extra embryonic mesoderm of the exocoelom (3) endodermal sinus structures with mantl ng of vessels by yolksac endoderm, (4) cystic structures lined by a layer of flat cells with protruding nucleus and continuous with the parietal lining of the endo dermal sinuses (Fig 1)

Autopsy Findings

Neither macroscopic nor microscopic signs of tumour tissue were observed in the mediastinum lymph nodes lung tissue, pericardium and heart A subphrenic clotfilled cavity from the operation was seen in the upper abdomen, reaching as far as the hepatic vein The remaining right liver lobe con tained metastatic tumour tissue of endodermal sinus tumour type The remainder of the intestinal tract, the retroperitoneal space and the urinary tract were normal The lower pole of the left testis showed a large, yellowish firm tumour, 2 cm in diameter, with cysts measuring from a few mm to 1 cm in diameter The pineal body was normal The histopathological picture of the tumour remains found in the liver corresponded to the picture in the operation specimen already mentioned The testis showed a preponderantly fibrous tumour with a suggestion of capsule formation and surrounding atrophic testis tissue Streaks of smooth musculature were seen in the connective tissue together with cysts covered by a mainly single layered epithelium of varying height No primary germ cells or endo dermal sinus structures were found

The histological preparations from the media stinal tumours removed 3 years previously, showed teratomata, partly with mature structures from all germinal layers partly tissue regions difficult to classify, but which did not contain endodermal sinus tumour elements

DISCUSSION

Endodermal sinus tumour is a rare malignant tumour form just described with extra gona dal localization only during the last 6–7 years The theoretical attempts to explain the extra gonadal localization of this tumour are either based on the assumption of migration of germ cells, with the possibility that they develop in time into germ cell tumours, or on the assumption of metastatic occurrence of primitive tumour forms from a primary tumour, assumed to be an adult teratoma, which should always be considered as potentially malignant

In these mature tumours, it is rare to find regions suggestive of primitive germ cell tumour Before maturation has been achieved, the primary teratoma is considered to have metastasized with germ cells, which in a suitable environment develop tumours of, for example, the endodermal sinus tumour type, choriocarcinoma, germinoma or teratoma

The case reported is remarkable for the finding of adult teratomata both with media stinal and gonadal localization The endodermal sinus tumour localized in the stomach may be a metastasis from a teratoma or may be a stage in a multiple teratoid anlage

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Acta path microb ol scand Section A 82, 182-188 1974

THE CALCIFIED CARTILAGE AND THE SUBCHONDRAL BONE UNDER NORMAL AND ABNORMAL CONDITIONS

J Stougård

The Research Laboratory, Orthopaed c Hospital Copenhagen, Denmark

The main object was to study the osteocartilaginous junction under normal and abnormal conditions from the femur and patella To this end the author used samples removed at autopsy from normal knee joints and biops es from patients with patellar chondromalacia Slight differences were found between non weight bearing and weight bearing areas of the joint These differences were accentuated in cases with mild cartilaginous changes mani festing themselves in increased thickening of the calcified cartilage and the subchondral bone, the junction of the two tissues growing more irregular and the number of bony extensions from the subchondral bone into the calcified cartilage increasing Th s ind cated increased activity in the skeletal tissue in the presence of cartilaginous changes as also confirmed by tetracycline labelling

The articular cartilage cannot be considered an isolated tissue, neither under normal nor under abnormal conditions Relations to neighbouring tissues, joints with synovial membrane and synovial fluid as well as to the undelying bone are extremely intimate, not least with respect to nutrition The purpose of the present study was to elucidate in more detail the junction between cartilage and bone i e *the area with calcified cartilage* and subchondral bone, by means of an ordinary histological technique, microradiography and after tetracycline labelling

METHODS

1 Decalcified specimens After decalcification in formic acid the specimens were dehydrated in alcohol embedded in paraffin and about 2-5 μ thick sections were cut and stained with haematoxylin eosin as well as toluidine blue

2 Un decalcified specimens The piece of bone was fixed in formaldehyde dehydrated with alcohol and embedded in methyl metacrylate Sections were cut with a rotating saw and ground to a thickness of 50 μ These sections were used for (a) Microradiography The picture was taken with a Machlett tube A E G 50 with a Wolfram anode using 12 kV and 10 mA at a focus distance of 15 cm and a exposure time of 10 minutes The film was a Kodak *spectroscopic plate* 649 0 and the *developer* D 19b

(b) Assessment of tetracycline labell ng in UV light us ng Riuchert f lter G G *9/1 mm* +O G *0/1 5 mm Thus treated* the labelled areas stood out *in* yellow clear fluorescence From these sections d apositives were prepared and magnified 25 × By a chartometer the labelled bony surfaces were measured to obtain a relat ve measure of the labelling

MATERIALS AND METHODS

The material as well as results fall into two groups

I Assessment of sections from weight bearing and non weight bearing articular areas on the femur and patella *removed post mor*

Received 14 vi 73 Accepted 1 x 73

tem from 2 females and 6 males aged 17–50 years Only knees without pathological changes and with grossly normal cartilage were used Nevertheless subsequent micro scopic examination revealed mild cartila ginous changes in about half the 64 samples. Fig 1 presents the 4 sites from where the samples were taken From each site 2 pieces of cartilage bone were removed with a saw, one centrally from the site presumed to carry most weight and one peripherally from a site where weight bearing had presumably been lighter

(a) The cartilage exhibited, despite its grossly normal appearance, microscopic signs of mild degeneration with superficial fibril lation and flake formation (grade I according to *Collins* 1949) in almost half the cases The thickness (measured with a graduated eye piece) varied from 1 0–4 2 mm somewhat greater on the patella and on the weight bearing areas as compared with the non weight bearing ones

(b) The calcified cartilage stained like the hyaline cartilage, but more faintly with to luidine blue The junction to the cartilage

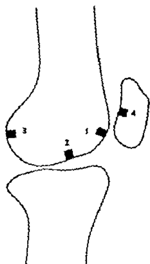


Fig 1 Sketch show s es on the femur and patella from where mples were taken one centrally and one pe rally on the joint surface from each area


Fig 2 Microradiography from a patella with normal cartilage At the top the calcified cartilage with an even junction to the hyaline cartilage (not shown in the picture) From the underlying subchondral bone and from the medullary cavity extensions are growing up into the calcified cartilage without reaching the hyaline cartilage (magn × 32)

the so called cement line was regular and approximately alike in the weight bearing and non weight bearing areas The continui ty might be interrupted by bony extensions (Fig 2) and cell clusters In several cases there was direct contact between bone and cartilage i e an area without calcified carti lage of a length up to 80 μ in both kinds of samples Direct contact between medullary cavity and calcified cartilage possibly sepa rated by a single layer of bone cells was ob served only in the femoral samples (Fig 3a + b) most often in the non weight bearing areas The length of such contact areas ranged from 30 to 250 μ The junction to the subchondral bone was considerably more ir regular and most irregular in weight bearing areas The thickness of the calcified cartilage was determined in each preparation as the mean of 5 values measured at a fixed mutual distance and given in Table 1 together with the maximum values found Higher values w r found in the patellar samples The va l es for the weight bearing areas were appro x n ately equal whereas marked fluctuations ere found among the non weight bearing reas This might be due to a certain variabi l ty in taking the samples as peripherally on

Acta path microbiol scand Section A 82, 182-188 1974

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(b) Assessment of tetracycline labelling in UV light, using Riuchert filter *G G 9/1 mm* +O G 0/1 5 mm Thus treated, the labelled areas *stood out in* yellow, clear fluorescence From these sections diapositives were prepared and magnified 25 × By a chartometer the labelled bony surfaces *were measured* to obtain a relative measure of the labelling

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Received 14 vi 73 Accepted 1 x 73

TABLE 1 *Thickness of the Calcified Cartilage and the Subcondral Bone*

Sample Site on Femur and Patella	Thickness of Calcified Cartilage (in micron) Mean Value		Maximum Value		Thickness of Subchondral Bone (in micron) Mean Value	
	Weight-bearing	Non-weight bearing	Weight-bearing	Non-weight-bearing	Weight bearing	Non weight-bearing
Femur 1	73	80	168	181	219	122
Femur 2	88	40	166	99	172	95
Femur 3	70	37	143	82	166	108
Patella 4	92	117	210	256	493	529

Thickness, in micron, of the calcified cartilage and subchondral bone from articular surfaces with normal hyaline cartilage on the femur and patella The numbers of the sample sites refer to Fig 1 From each site two samples were taken, one centrally, named weight bearing, and one peripherally, named non-weight bearing



Fig 4 Microradiography from a patella with moderately severe cartilaginous degeneration There is thickening of the calcified cartilage which exhibits several parallel lines (cement lines?) (magn × 32)



Fig 5 Microradiography from a patella with moderately severe cartilaginous degeneration Thickening of the calcified cartilage and of the subchondral bone Deep in the bone islets of calcified cartilage (magn × 32)

lage on the femur and tibia, but cartilaginous changes on the patella, in 2 patients so pronounced that the bone was denuded As the present study was designed to investigate the appearances in moderate cartilaginous changes these two patients were excluded With a cylindrical drill biopsies were taken from patellar areas with degenerated cartilage and, for comparison from non weight bearing areas with normal cartilage on the patella and femur

On the microradiograph from patellae with hyaline cartilage lesions the calcified cartilage proved to be intact throughout Thus, apart from bony extensions, there was no direct contact between the medullary cavity-bone and the hyaline cartilage The junction to the latter was even, with an intact cement line Where the calcified cartilage was particularly thick there were several parallel bands similar to the named cement line (Fig 4) The junction to the subchondral bone was considerably more irregular than in the normal biopsies

The thickness of the calcified cartilage varied within wide limits, the mean values



Fig 6 Fluorescence micrograph from a patella with moderately severe cartilaginous degeneration labelled preoperatively with tetracycline There is considerably pronounced labelling and double labelling, mostly in the subchondral bone Also pronounced labelling of the cement line (magn × 32)

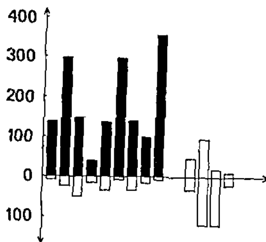


Fig 7 The relative labelling of bone surfaces from the femur and patella of patients preoperatively labelled tetracycline Each column represents a patient Below the abscissa values from the femur Above the abscissa values from the patella, those from areas with abnormal cartilage in black Values from areas with normal cartilage, from the femur as well as patella, in white

ranging from 146 to 292 μ, average 219 μ The maximum thickness varied even more, from 175 to 584 μ

The subchondral bone gave an impression of a considerably more condensed bony structure The junction to cancellous bone was even more difficult to make out At an estimate the thickness was twice that of the normal weight-bearing patellar biopsies There was an increased number of islets of calcified cartilage and deeper in the bone than in the normal biopsies Possibly this was related to the considerably more irregular junction between the two tissues

Labelling, and also double labelling, with tetracycline was found, in UV light, to be localized chiefly in the subchondral bone, most distinctly just basal to the calcified cartilage (Fig 6) In about 10 per cent the labelling was more diffuse in the bony tissue The degree of labelling is depicted in Fig 7 Each column represents a patient, the results from the patellar biopsies plotted above the abscissa and those from the femur below There was a somewhat more pronounced labelling of the normal patellar biopsies as compared with the femoral biopsies, possibly because of the denser skeletal structure

Biopsies from areas with cartilaginous degeneration showed a considerably mor pronounced labelling The most strongly labelled preparation (length of labelling 360 versus the normal average of 74 measuring units) was from a bipartite patella in which cartilaginous changes were present on the patella as well as on the extra bone The double labelling was also most pronounced in this preparation The labelling was not localized exclusively to bony tissue A certain amount of autofluorescence was seen in the hyaline cartilage, but not in the calcified cartilage which, on the other hand, showed labelling of bony ex-

tensions The cement line and the parallel bands were in several cases strongly labelled

DISCUSSION

According to *Collins* (1949) the calcified cartilage constitutes the oldest layer of cartilage, acting as an impenetrable barrier, so that direct contact between bone and hyaline cartilage is considered an abnormal and unstable condition In rabbits *Holmdahl & Ingelmark* (1950) demonstrated a direct contact between bone medullary cavity and hyaline cartilage via canal-like structures (dendritic) and a wider contact (ampulla) *Ingelmark* (1950), *Ekholm* (1955) and *Trueta* in several studies, the most recent one from 1968 concerning the findings in the patella, found that the basal third of the cartilage was nourished by the underlying bone In the present material normal biopsies showed canal like contacts up through the calcified cartilage as areas with wider contact between bone medullary cavity and the hyaline cartilage The latter contact, however, could not be demonstrated in the abnormal biopsies

A comparison of weight bearing with non-weight bearing articular areas showed, in the case of the femoral samples, a considerably more irregular junction between the calcified cartilage and the bone and twice as many bony extensions into the calcified cartilage in the weight-bearing areas This difference was not demonstrable in the patella which, however, exhibited an increase in the number of bony extensions of 50 per cent as compared with the weight-bearing femoral areas The thickness of the calcified cartilage differed within wide limits It was least in the non weight bearing femoral areas, greatest from patellar areas with cartilaginous degeneration, showing mean values of 219 μ (as compared with 104 μ from weight-bearing and non weight bearing areas on patellae with normal cartilage) The same has been reported by *Bennett et al* (1942) and by *Trueta* (1968) whereas *Green et al* (1970) found no such thickening of the calcified cartilage, which they studied particularly on the patella

The cement line, viz the junction between the hyaline and the calcified cartilage, was approximately alike in the normal and in the abnormal preparations, but exhibited a considerably more pronounced labelling with tetracycline in the latter The named parallel bands have previously been described in osteoarhritis by *Trueta* (1968) and by *Green et al* (1970) The latter workers also found them in changes of the subchondral bone and on "intact patellae" in elderly persons

The subchondral bone, fairly well defined in the femur, less well defined in the normal patellar biopsies, and least well defined in the abnormal patellar biopsies—exhibited marked variations in thickness, from 100 μ in the non-weight bearing femoral biopsies to about 1000 μ in the abnormal patellar biopsies *Parker et al* (1934), *Bennett et al* (1942), *Collins* (1949), *Wiles et al* (1956) and *Trueta* (1968) have made similar findings However, according to *Bennett et al* and *Collins* a normal subchondral bone might be encountered even in cases with severe cartilaginous changes Islets of calcified cartilage were found in the subchondral bone, of a deeper situation and in greater numbers in the abnormal preparations These islets might be imagined to have appeared due to perpendicular cutting of highly branced extensions of calcified cartilage down into the subchondral bone *Parker et al* (1934) as well as *Bennett et al* (1942) believed they had formed by metaplasia of the medullary tissue

The question whether the cartilaginous changes on the patella, clinically known as patellar chondromalacia, are tantamount to incipient osteoarthritis cannot be solved here *Darracott et al* (1971) considered patellar chondromalacia to be different from osteoarthritis, starting in the skeletal tissue, possibly of vascular origin and they found radiological as well as histological evidence of osteoporosis and narrowing of the subchondral bone These apparently widely different changes and views might be imagined to be due to the degree of cartilaginous changes

That increased skeletal activity takes place even in the presence of modest cartilaginous changes is apparent from tetracycline-labelled preparations The labelling and the double labelling were considerably more pronounced in patellar preparations with cartilaginous changes and localized most distinctly in the subchondral bone close to the junction to the calcified cartilage

CONCLUSION

Samples from the femur and patella with normal cartilage showed differences in the calcified cartilage and in the subchondral bone between articular areas subjected to different weight bearing Some of these differences were accentuated even by mild cartilaginous changes as seen on the patella Both tissues increased in thickness, the junction between them grew more irregular, and the number of bony extensions from the subchondral bone into the calcified cartilage increased These signs of increased activity in the skeletal tissue were further confirmed by tetracycline labelling

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Acta path microbiol scand Section A 82, 189–198, 1974

AN *IN VITRO* STUDY OF CYTOSTATIC DRUG EFFECT ON THE DNA SYNTHESIS IN METHYLCHOLANTHRENE INDUCED MOUSE SARCOMAS. CORRELATION BETWEEN *IN VITRO* RESULTS AND THE RESPONSE *IN VIVO*

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The present work uses an *in vitro* test model to study the effect of vinblastine sulphate, melphalan and cytosine arabinoside on the DNA synthesis of methylcholanthrene induced mouse sarcoma With this model, we studied the possible correlation between the *in vitro* results and the response to these cytostatic drugs *in vivo* Seven different tumours were tested and at the same time the tumour cell suspension was transplanted to a number of recipients The *in vitro* effects of the drugs were measured as differences in incorporation of labelled H^3 thymidine in drug containing tubes and in control tubes The effects of the drugs on the methylcholanthrene induced sarcomas varied greatly, from strong sensitivity to resistance Two days after transplantation cytostatic drug treatment began and continued for 10 days Its effect was checked with white blood cell counts The effect on the *in vivo* growth of the transplanted tumours was evaluated by registration of the number of palpable tumours on different days in each experimental group A correlation was found between the *in vitro* test results and those of *in vivo* treatment Its biological significance is discussed

Many different *in vitro* techniques have been used to study tumour cell susceptibility to cytostatic drugs with the hope of obtaining reliable prediction tests for the *in vivo* response (16 11) The present authors (7) used a short term incubation technique where a tumour cell suspension was exposed to a cytostatic drug for four hours, the DNA synthesis during the last hour being used as a parameter of tumour cell susceptibility The method was evaluated on methylcholanthrene-induced mouse sarcoma It was possible to characterize different tumours and also to study the phenomenon of heterogeneity within the tumours and changes in reactivity during serial transplantation (8, 9) The parameter studied—suppression of tritiated thymidine incorporation into DNA—was markedly influenced by the presence of cytostatic drugs in some tumour cell suspensions, in others, no effect was seen Many reasons can be suggested for why this *in vitro* response should not mirror the *in vivo* susceptibility of the tumour cells to drug treatment comparatively high drug concentrations were used compared to the serum concentrations ob-

Received 22 1 74 Accepted 22 1 74

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tained *in vivo* at pharmacotherapy, DNA synthesis was used for evaluating drug effects also when the drug primarily attacks other cellular *processess, immunological* phenomena, vascularization, and other factors of importance for the *in vivo* growth of a tumour do not interfere in the test, etc.

The present study was devised to further investigate the observed variability of *in vitro* susceptibility to cytostatic drugs, as evaluated by the method used. The object was to investigate whether any relation exists between the *in vitro* response and the behaviour of the same *tumour cell suspension during drug* therapy *in vivo*.

Seven tumours were tested *in vitro* with the above-mentioned short-term incubation method. At the same time, the tumour cell suspension was transplanted to a number of syngenic recipients. Two day after transplantation, treatment of the mice with cytostatic drugs began and continued for 10 days. Tumour appearance was evaluated every third day for 18 days. The capacity of the drugs to suppress tumour development was correlated to the *in vitro* response.

MATERIAL AND METHODS

Animals. Female syngenic C_5 black mice, average body weight appr. 20 g, obtained from G. L. Bomholtgard (Ry, Denmark), and kept on standard laboratory food and water *ad lib.*

Tumour material. Methylcholanthrene was injected subcutaneously on the back, 50 µg being given in 0.1 ml sesame oil. Tumours with a histo*logical structure of fibrosarcoma developed after* 3 months in about 80 per cent of the animals. Five *animals were chosen for the experiment.* The tumour had then grown for three to four months. To obtain sarcomas resistant to cytostatic drugs two were transplanted serially and the hosts treated *in vivo* with cytostatic drugs. One sarcoma (called S 6) was serially transplanted during twelve generations (12 months) and the host animals were given 2.5 mg/kg body weight of melphalan twice a week. Another sarcoma (S 7) was treated in the same way, but vinblastine sulphate was given at 1.0 mg/kg body weight. In the 12th generation both sarcomas were practically resistant *in vitro* to the drugs used during treatment.

In vitro determinations of susceptibility to drugs. The detailed description of the method is given in (6, 7). A cell suspension is prepared from the tumour. Part is used for transplantation purposes (see below), part is distributed into a number of test tubes in a serum free medium consisting of Parker 199 (SBL, Stockholm). The tubes are divided into four groups: one control and one group for each cytostatic drug tested; three tubes in each group. The cells were preincubated with the drugs for 3 hours. Final concentrations of cytostatic drugs: melphalan—200 µg/ml, vinblastine sulphate—100 µg/ml, cytosine arabinoside—250 µg/ml.

After the preincubation period ended, H^3 thymidine (H^3 TdR, methyl H^3 thymidine, Schwartz Mann, Orangeburg, spec. act. 1.9 Ci/mM) was added to a final concentration of 2 µCi/ml and *incubation was continued for a further hour.*

The cells were then washed once with Parker 199; nucleosides and nucleotides were extracted with cold 5 per cent trichloroacetic acid (TCA) and the cell residue was rinsed with TCA and with ethanol and then dissolved in 0.6 ml of 1 N sodium hydroxide.

Radioactivity of the solution was determined from a 100 µl sample dissolved in 1 ml Soluene (Packard Ltd.) and mixed with 14 ml scintillation fluid (0.3 g dimethyl POPOP, 5 g PPO, 1 000 ml toluene). Counting was done for 10 minutes in a Packard Tricarb 3310 spectrometer with external standardization. Two further samples, 200 µl each, were used for determination of DNA with the indole method of Ceriotti (3) as modified by Bonting and Jones (2). The value obtained was corrected with the aid of a standard curve using calf thymus DNA to an adjusted DNA value.

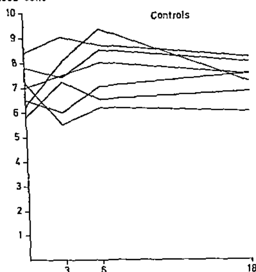
The thymidine uptake in a cell sample is evaluated as the following expression which results in near normally *distributed variates*:

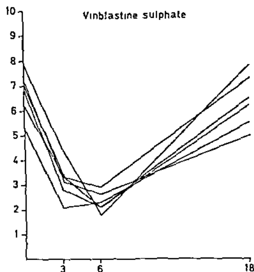
$$a = 100 \times \log_{10} \frac{\text{c.p. 10m} \times 10^4}{(\text{AES}) \times (\text{DNA})}$$

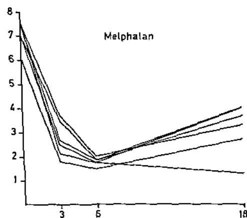
where (AES) represents the c.p.m. registered with the automatic external standardization and (DNA) the mean of the two adjusted DNA values as determined above. For a discussion of this formula see (7) and (13).

The effect of a certain cytostatic drug *in a* specific experiment is evaluated as the difference between the mean *a* value of the three control tubes and the mean *a* value of the three tubes that the cytostatic drug was added to.

Transplantation method. From the cell suspension prepared for the *in vitro* test, a small volume was injected *subcutaneously on the flank of* each recipient. Each injection volume was 0.1 ml and contained 5 × 10^4 *cells.* As described previously (7) this *transplantation* method results in almost 100 per cent takes of tumours at all injection sites within a fortnight.







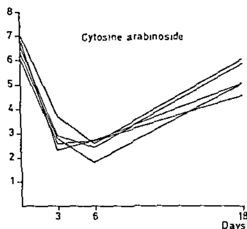


Fig 1 White blood cell count before, during, and after drug treatment (begun on day one and ended on day ten) Day one in the diagram represents the first day of treatment No of white blood cells given as thousands

Treatment of recipients with cytostatic drugs Two days after the transplantation of the tumour cell suspension treatment of the recipient mice began and continued for 10 days The drugs were given intravenously in a tail vein control mice being similarly injected with Parker 199 The amounts of drugs given were melphalan 2 5 mg/kg body weight vinblastine sulphate 1 0 mg/kg body weight cytosine arabinoside 50 mg/kg body weight Injected volume was 0 1 ml

Control of WBC To check the *in vivo* effect of the cytostatic drugs white blood cell count (WBC) was followed before, during (day 3, 6), and after the drug treatment (day 18) Blood samples were taken from the retrobulbar vein plexus and standard WBC was made In control mice and in pretreatment stages, WBC varied from 5 000 to 10,000 cells/mm³ During treatment with melphalan, from 1,500 to 3,500 with vinblastine sulphate from 2 000 to 3,000 and with cytosine arabinos de from 1,500 to 3,500 cells/mm³ At the end of the experimental period, WBC had returned to near normal values in animals treated with vinblastine sulphate or cytosine arabinoside, the WBC for the melphalan treated groups was still depressed (Fig 1)

TABLE 1 In vitro *Effects of Cytostatic Drugs on the Seven Sarcomas*

Number of tumours	Vinblastine sulphate effect	melphalan effect	cytosine arabinoside effect
Sarcoma 1	15NS	36†	8NS
Sarcoma 2	26§	30†	22*
Sarcoma 3	88†	56†	74†
Sarcoma 4	85†	85†	92†
Sarcoma 5	110†	38†	110†
Sarcoma 6	43†		
Sarcoma 7		–20NS	

* = 0 05>p>0 01, § = 0 01>p>0 001, † – p<0 001
NS = non significant
Effect expressed as difference between mean control value and mean value with cytostatic drug S gnifi cance levels tested with analysis of variance, comparing ' between drug and control with the common error variance (cf 7)

TABLE 2 *Effect of Cytostatic Drugs on the* in vivo *Growth of the Transplanted Tumours as Evaluated by Registration of the Number of Animals with a Palpable Tumour Present at Different Days in Each Experimental Group*

Sarcoma No	*In vivo* treatment	days 6	9	12	15	18
S 1	control	4/8	6/8	7/8	7/8	7/8
S 1	vinblastine sulphate	2/8	3/8	5/8	6/8	8/8
S 1	melphalan	1/8	2/8	2/8	3/8	6/8
S 1	cytosine arabinoside	3/8	7/8	7/8	8/8	8/8
S 2	control	4/6	5/6	5/6	5/6	5/6
S 2	vinblastine sulphate	0/6	1/6	1/6	1/6	1/6
S 2	melphalan	1/6	2/6	2/6	2/6	2/6
S 2	cytosine arabinoside	2/6	4/6	5/6	6/6	6/6
S 3	control	5/15	10/15	13/15	15/15	15/15
S 3	vinblastine sulphate	0/15	1/14	2/14	3/14	6/14
S 3	melphalan	0/15	2/15	3/15	5/15	6/14
S 3	cytosine arabinoside	0/15	2/15	6/15	8/14	13/14
S 4	control	8/15	14/15	15/15	15/15	15/15
S 4	vinblastine sulphate	1/14	2/14	3/14	4/14	5/14
S 4	melphalan	2/15	3/15	4/15	6/15	7/15
S 4	cytosine arabinoside	1/14	4/14	6/14	9/14	12/14
S 5	control	8/12	11/12	12/12	12/12	12/12
S 5	vinblastine sulphate	0/12	0/12	1/12	1/12	3/12
S 5	melphalan	4/12	4/12	5/12	7/12	7/12
S 5	cytosine arabinoside	1/11	2/11	4/11	5/11	7/11
S 6	control	11/15	13/15	15/15	15/15	15/15
S 6	vinblastine sulphate	3/15	7/15	11/15	14/15	15/15
S 7	control	6/15	12/15	14/15	15/15	15/15
S 7	melphalan	5/15	11/15	13/15	13/15	15/15

RESULTS

In vitro *Results*

Table 1 records the results of the *in vitro* tests of the seven studied sarcomas It shows that the tumours differed in response to each of the three cytostatic drugs

In vivo *Results*

The effect of cytostatic drugs on the *in vivo* growth of the transplanted tumours was evaluated by registration of the number of animals with a palpable tumour present at different days in each experimental group

Fig 2 Growth curves i e the percentage of takes at each day shown for control animals and animals treated with the three cytostatic drugs S 1 7 mark sarcoma number Day one in diagram represents the first day of treatment

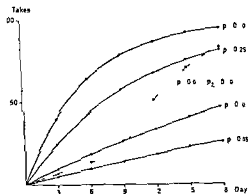


Fig 3 Theoretical growth curves (cf Fig 2) at different p values

Table 2 gives the numbers observed in the seven series In Fig 2 the growth curves, i e the percentage of takes at each day are shown for control animals and those treated with the various cytostatic drugs During the period of drug treatment i e up to day 10 in the diagrams all drug treated groups show a depressed tumour growth except S 1, and possibly also S 2 when treated with cytosine arabinoside and S 7 when treated with mel phalan. After day 10 the curves behave differently In most instances growth ap parently continues more or less unchanged compared with the early growth period per haps with a slight acceleration Tumours S 3–S 5 when treated with cytosine arabinoside show a spurt of growth activity after the end of the treatment period and a slight but similar effect can also be seen in S 2 In S 3 for instance a near 100 per cent take was obtained after 18 days with cytosine arabino side treatment despite the depression of tu mour growth during the treatment period

TABLE 3 *Comparison of the Mathematical Model Used for Evaluation of Tumour Growth* in vivo *and Data for 86 Graft Sites in Control Animals, Transplanted with Material from Seven Original Tumours (S 1–S 7)*

A) *Common p value (p 0 40)*

Tumour No	Day 6 Found	Day 6 Exp	Day 9 Found	Day 9 Exp	Day 12 Found	Day 12 Exp	Day 15 Found	Day 15 Exp	Day 18 Found	Day 18 Exp	Own p value
S 1	4	5 1	6	6 2	7	7 0	7	7 4	7	7 6	0 34
S 2	4	3 8	5	4 7	5	5 2	5	5 5	5	5 7	0 40
S 3	5	9 6*	10	11 7	13	13 1	15	13 8	15	14 3	0 32
S 4	8	9 6	14	11 7	15	13 1	15	13 8	15	14 3	0 45
S 5	8	7 7	11	9 4	12	10 4	12	11 0	12	11 4	0 49
S 6	11	9 6	13	11 7	15	13 1	15	13 8	15	14 3	0 51
S 7	6	9 6	12	11 7	14	13 1	15	13 8	15	14 3	0 37

*χ^2 = 6 12 0 025>p>0 01

B) *Separate analysis of S 3 (p = 0 32) and the other six tumours (p 0 42)*

Tumour No(s)	Day 6 Found	Day 6 Exp	χ	Day 9 Found	Day 9 Exp	χ^2	Day 12 Found	Day 12 Exp	χ^2	Day 15 Found	Day 15 Exp	χ^2	Day 18 Found	Day 18 Exp	χ^2
S 3	5	8 1	2 6	10	10 3		13	11 8		15	12 9	–	15	13 5	
S 1 2 4 7	41	46 9	2 2	61	56 8	1 6	68	63 2	3 3	69	66 0	1 9	69	68 2	–

To evaluate these changes quantitatively, the following mathematical model was used

During each 3 day period, a certain fraction, p, of all tumours reach a palpable size Suppose that p is constant during a sequence of i 3 day periods During the first 3 day period—up to day 3—pN animals should show a tumour N being the number of animals injected During the next 3-day period—up to day 6—another $pN(1-p)$ should appear, etc After i periods, $N[1-(1-p)^i]$ animals should carry a tumour

The graphs in Fig 3 illustrate the number of expected tumours for each three-day period at different p values The general form of the graphs agrees fairly well with those actually found in control animals (cf Fig 2) Given a certain p value, the expected number of tumours each day in each experiment can be calculated By determination of the expression

$$U = \Sigma \frac{(\text{found No} - \text{expected No})^2}{(\text{expected No})}$$

we can estimate how well the model fits the actual material For each material, a p value can be calculated which gives a minimum value of U

To evaluate this model, the total material of grafts to untreated animals is used It comprises 86 graft sites from seven different tumour sources (S 1–S 7) Table 3 summarizes the result For the total material, a minimum U value is obtained for $p = 0\,40$ Expected and found numbers of tumours at this p value are shown, there is a significant deviation for one tumour, S 3, with too few tumours developed at day 6 The Table shows the expected and found numbers for each day for the S 3 tumour at a p value calculated for it alone $p = 0\,32$, and also the corresponding values for the remaining six tumours, using a p value calculated for these alone, $p = 0\,42$ There is no significant deviation between expected and found numbers of tumours at any day The model appears to give an adequate description of the growth and can apparently discriminate between the growth capacity of different tumours

When tumours are studied in animals that had received cytostatic drugs during the first three 3-day period, a change in growth rate—and therefore also in p value—could occur after day 10 In Fig 3, such an example can be seen (the dashed graph) During the first 10 days, a reduced growth rate of $p_1 = 0\,07$ was presumed followed by a higher rate during the later part of the observation period with $p_2 = 0\,40$ A graph is thus obtained resembling that found for certain cytostatic drugs, e g , S 1 treated with melphalan (Fig 2)

The expected number of tumour-bearing animals after growth during cytostatic drug treatment for i 3-day periods followed by j 3 day drug free periods will then be $N[1-(1-p_1)^i(1-p_2)^j]$

Again, values for p_1 and p_2 are chosen which give a minimum value of U

This has been done for all tumours and all types of treatments studied Table 4 gives the results For the drug treated groups, two values are given the p_1 and p_2 values which give a minimum deviation, and the common $p = p_1 = p_2$ value which gives a minimum For each tumour and treatment, the most reasonable value was chosen a common p value if p_1 and p_2 do not differ markedly or if p_2 is smaller than p_1 When large differences are present between p_1 and p_2 and p_1 is smaller than p_2 these values are kept instead of a common p value

To study a possible correlation between *in vitro* effects and *in vivo* effects the p values are compared with the *in vitro* determinations of drug effects The latter (see Table 1) have so far been expressed as $\log_{10}$ differences For the purpose of graphically demonstrating the effects they are transformed to the percentage reduction of control values Fig 4 shows the result of the plot This way of presenting the data gives a better visualization of the effects than the differences in *a* values, but it also results in an increased dispersion at high values and a decreased dispersion at low values

Fig 5 shows a similar plot between the *in vitro* test result and the p_1 values Seven tu

TABLE 4 *Estimated* in vivo *Growth Rate of Transplanted Tumours*

Sarcoma number	Control	Vinblastine sulphate			Melphalan			Cytosine arabinoside		
	p	p	p_1	p_2	p	p_1	p_2	p	p_1	p_2
1	*0 34*	0 21	*0 14*	*0 44*	0 11	0 08	0 20	0 37	*0 33*	0 66
2	*0 40*	*0 04*	0 05	0 01	*0 09*	0 12	0 01	0 32	*0 25*	*0 70*
3	*0 32*	0 05	*0 02*	*0 12*	0 07	*0.04*	*0 15*	0 14	*0 04*	*0 40*
4	*0 45*	0 06	*0 04*	*0 10*	0 09	*0 07*	*0 13*	0 15	*0 08*	*0 33*
5	*0 49*	0 03	*0 01*	*0 07*	0 15	0 14	0 13	0 10	*0 05*	*0 20*
6	*0 51*	0 25	*0 15*	*0 58*						
7	*0 37*				0 31	*0 27*	*0 58*			

The table gives values of p, p_1, and p_2 resulting in minimum deviations from found values of tumour frequency Values in italics were chosen for growth rate characterization Further explanations in text

mours—four treated with cytosine arabinoside, two with vinblastine sulphate, and one with melphalan—apparently returned to the same or higher growth rates than those present in the control animals with a *p* value between 0 33 and 0 70 The remaining tumours apparently continue growth almost as slowly as during the treatment period

DISCUSSION

The main finding of the present investigation is that a correlation exists between the *in vitro* susceptibility of a tumour cell suspension to a cytostatic drug and the *in vivo* effect of the same drug on the same tumour cells evaluated with the method used ability of the drug to suppress tumour development after transplantation Despite the many objections raised in the introduction against *in vitro* methods of the type used, the *in vitro* test apparently gives a result with a certain degree of significance related to *in vivo* tumour growth This does not mean that the parameter studied *in vitro*—DNA synthesis—is necessarily a target for the *in vivo* tumourostatic effect of the drug involved To illustrate this, it can be mentioned that vinblastine to a great extent exerts its action as a mitotic poison and not as a DNA synthesis blocker, even though an effect on DNA synthesis has been demonstrated (14) A correlation between

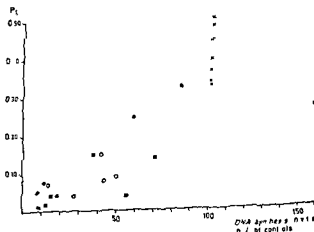


Fig 4 Correlation between growth rate (p_1) *in vivo* during treatment with a cytostatic drug and DNA synthesis *in vitro* in test with the same drug
Designations as in Fig 2

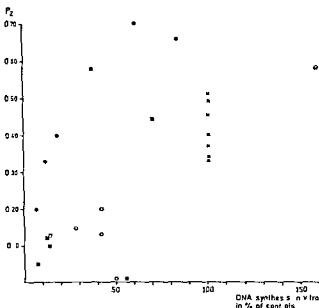


Fig 5 Correlation between growth rate (p_2) *in vivo* after the end of the treatment with a cytostatic drug and DNA synthesis *in vitro* in test with the same drug Designations as in Fig 2

the *in vitro* response as DNA synthesis depression and the *in vivo* suppression of tumour development can depend on tumour characteristics regulating e g, uptake or intracellular degradation of the drug The *in vitro* method thus apparently estimates a tumour cell characteristic which has a biological meaning also for *in vivo* conditions This does not mean that the test can be applicable for prediction tests in other situations

The mode of estimating *in vivo* susceptibility is relevant only for the effect of the drugs in question on an inoculum of tumour cells and might not be relevant for the effect of manifest tumour growth Factors such as immunological response and tumour vascularization do not enter the picture during the initial days of tumour growth studied in this way In the present study, the same tumour cell suspension was investigated *in vitro* and *in vivo* When *in vitro* tests are applied as prediction test in a clinical situation, the material for the *in vitro* test can represent only a small part of the total tumour mass to be treated *in vivo*—the problem of tumour heterogeneity then becomes important (cf 8, 9)

Many reports on *in vitro* tests for cytostatic sensitivity dealt with human tumours, this made the evaluation of the *in vivo* results very dificult The correlation described in these reports is therefore often uncertain (cf 1, 5, 12, 17, 18, 19, 20) The situation is somewhat better when human leukemic cells are tested *in vitro* A relatively good correlation between *in vitro* and *in vivo* cytotoxic effect has been found, but the predicting value of the *in vitro* tests for obtaining haematological remission was low (4) Animal tumours have been used in some reports on correlation between *in vivo* and *in vitro* results Seidel & Wegner (15), for instance, reported a relatively good correlation, but they never tested the effect of the same drug *in vivo* on tumours sensitive and on tumours resistant to the drug *in vitro*

This work has been supported by grants from the *Faculty of Medicine*, University of Lund, and the *John and Augusta Persson Foundation*

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Acta path microbiol scand Section A 82, 199 210, 1974

RENAL LESIONS IN RATS WITH LONG-TERM ALLOXAN DIABETES

A Semiquantitative Light Microscopic Study with Particular Reference to the Glomeruli

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A light microscopic quantitation and comparison of some selected glomerular structures (basement membrane and mesangial area) in nondiabetic and in untreated alloxan diabetic rats at various ages was performed It applies to both groups of rats that a significant age related increase of graded glomerular basement membrane thickness and of measured mesangial area developed In time, however this increase was significantly more pronounced in the diabetic than in the nondiabetic group Tubular and interstitial changes were also observed both in nondiabetic and in diabetic rats The importance of using an adequate number of nondiabetic age matched controls in studies of the mentioned glomerular changes in diabetic rats is emphasized A quantitation of the changes in blind studies is advisable

The pathogenesis of small vessel disease in human diabetes is still obscure Studies of microvascular changes which occur in diabetic animals are therefore desirable, such studies will make it possible to use homogenous groups of animals to observe them daily under known standardized conditions and to study the influence of different therapeutic measures on the course of the changes Furthermore if animals in which diabetes is induced experimentally are used, genetic factors which may be of importance for the development of the microangiopathy in human diabetes are practically excluded

There are several publications reporting a progressive periodic acid Schiff-(PAS)-positive thickening of the glomerular capillary walls (or of their basement membrane) and/or widening of the mesangial regions, in rats with experimental diabetes, usually induced by alloxan injection (9,10,14,21,22, 26) Results obtained in nondiabetic controls are either lacking (14), incompletely presented (10,21), or the kidneys of these animals have been classified as normal or without appreciable lesions (9,22,26) In other reports, age related glomerular changes in nondiabetic rats similar to those occurring in diabetic rats have been described (3 4, 8) The results mentioned above are based on general assessments and not on quantitations of the various structures Tubular and interstitial lesions both in nondiabetic and diabetic rats have also been reported (*cf* 4, 33)

The main purpose of the present report is to present the results of a light microscopic, quantitative and comparative study of glo

Received 3 vii 73 Accepted 9 x 73

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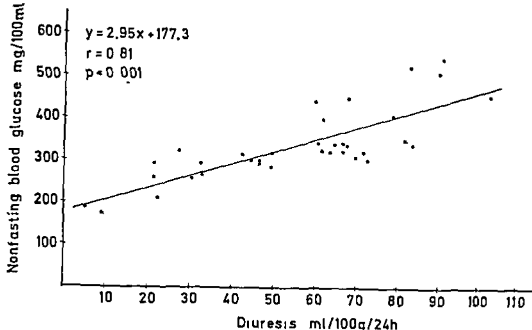


Fig 1 Correlation between weight adjusted urinary output and nonfasting morning blood glucose level in 35 alloxan diabetic rats of both sexes with varying durations of diabetes (1–12 months) Each blood glucose value is the mean of a sample taken immediately before and after the 24 hour urine collection period

merular basement membrane thickness and of mesangial area in nondiabetic and in alloxan diabetic rats at different ages These results will serve as the basis for different therapeutical regimens to be applied to alloxan diabetic rats

MATERIAL AND METHODS

Altogether 345 animals were investigated They were albino rats of both sexes of the highly inbred R strain kept at the Department of Cell Research, Karolinska Institutet, Stockholm All animals were maintained in air-conditioned rooms at 21–22° C on regular laboratory diet (pellets for rats and mice, Astra Ewos AB, Sodertalje) and were given tap water *ad libitum* The rats were housed in large plastic cages, about 3–4 rats per cage

Alloxan diabetic rats (160 animals) At the age of 3 months, the rats were starved for 48 hours Laparotomy was performed, using ether anaesthesia, and a single injection of alloxan (alloxan monohydrate, Eastman Kodak Co, Rochester N Y), 55 mg/kg, was given into the inferior caval vein The renal pedicles were clamped during the injection and for 5 minutes after (26) No insulin was given afterwards

Once a month the animals were weighed and moved to metabolic cages for quantitative collection of urine The 24 hour urine output was measured and qualitative tests for glucose (Clinistix®, Ames Company) and ketone bodies (Ketostix®, Ames Company) in the urine were performed Nonfasting morning blood glucose levels (from blood samples from the tail tip), and quantitative urine glucose values were determined intermittently (by the glucose oxidase method, using GLOX®, KABI, Stockholm), usually about 2 weeks as well as 6 and 12 months after alloxan injection Since a significant, linear relationship ($r = 0.81$, $p < 0.001$) between the blood glucose level and weight adjusted 24 hour urine output was found (Fig 1), the mean of weight adjusted urine volumes during the life span of each rat was considered to be a fairly accurate picture of its diabetic state All rats were found to be permanently diabetic during the entire period of observation They had a fairly constant urine output with a mean of at least 20 ml/100 g/24 h, corresponding to a blood glucose level of at least 200 mg/100 ml (Fig 1) Ketonuria was not observed Diabetic rats consumed about 15–18 g/100 g of food daily

Control rats 79 of the rats were treated exactly in the same way as the alloxan injected animals at 3 months of age except that saline was injected instead of alloxan The rest of the control rats (106 animals) were left untreated The nondiabetic rats appeared to be healthy without polyuria (urine

output was always less than 10 ml/100 g/24 h) or glucosuria Nonfasting blood glucose level, if con trolled (in most of the rats), was always less than 120 mg/100 ml (usually less than 100 mg/100 ml) Daily food consumption was about 5 7 g/100 g per rat

Renal biopsy At the ages of 4, 6, 9, 12 and 15 months, corresponding to a diabetes duration of 1, 3, 6, 9 and 12 months respectively various groups of animals were anaesthesized with ether and lapa rotomized The kidney pedicle on one side was clamped and a wedge shaped biopsy specimen (about 2 × 3 × 6 mm) was taken from that kid ney Blood for urea N and creatinine determina tions were withdrawn by heart puncture The ani mals were usually killed after that procedure but in some cases (45 out of 231 rats that underwent kidney biopsy) the abdominal incision was sutured and the animals were kept alive for further bio psies (1 3 repeated biopsies)

The kidney specimens were processed for paraffin embedding mainly according to the method of *Sainte Marie* (30) All specimens were cut into sections of 5 μ and stained with the PAS procedure (*cf* 19) Most of the sections were also stained with van Gieson's stain and subjected to a conven tional histopathological examination in order to investigate whether any intercurrent diseases oc curred in any of the rats The PAS stained sections were coded and studied at a magnification of about 400 × (using objective 40 × of the light microscope)

Semiquantitative grading of glomerular basement membrane thickness All PAS stained specimens were used in this study The thickness of the PAS positive basement membrane was assessed in 4 grades (1+, 2+ 3+ and 4+) (Figs 2, 3, 4 and 5) Each section consisted of about 40 80 glome ruli, all of which were studied and the average graded basement membrane thickness for each spe cimen was recorded A similar semiquantitative grading of skin vessel walls has previously been performed by *Save-Soderbergh et al* (31) and *Larsson* (18)

Quantitative determination of mesangial area by point counting method All biopsies taken from rats at the age of 4 months and most of those taken from 15 month old rats were sectioned at 3 μ and stained with the PAS procedure for measuring the mesangial area (13) The material was coded and measurements were performed at 512 × (objective 40 ×) with point-counting method with aid of an ocular grid The distance between the latt ce lines was 10 μ The sections were investigated systema tically and the first 10 intact glomeruli of each section with a minimum glomerular area of 100 points were measured Almost all measured glo meruli (over 99 per cent) were less than 300 points Each selected glomerulus was adjusted to the middle of the visual field The total corpuscular area within Bowman's capsule was determined and then the mesangial area was measured twice and the mean recorded The relative mesan gial area of each glomerulus was calculated in per cent of the total corpuscular area The mean me sangial area of the 10 measured glomeruli of each rat was then computed Ten sections were random ly selected and remeasured The variation coeffi cient of the double measurements was 10 0 per cent

Assessment of tubulo interstitial changes This was performed according to a semiquantitative 4 point scale 0, 1+ (slight), 2+ (moderate) and 3+ (severe) changes

Laboratory investigations These were performed on rats that had not undergone previous kidney biopsy Serum urea N was determined by urease and the *Berthelot* reaction (12) Serum creatinine was assayed, using *Lloyd s* reagent as absorbing agent (12) In some male rats urine albumin was determined by the *Lowry* method (20), preceded by protein precipitation in trichloroacetic acid and solubilization of albumin in ethanol (16) For albu min determinations, urine was collected during 24 hours using starving rats in order to obtain a more concentrated urine

Statistical methods Graded glomerular basement membrane thickness was analysed, using the non parametric rank sum test of *Wilcoxon* (35) For comparisons of measured mesangial area and of laboratory tests Student s t test was used All tests were performed at the 5 per cent significance level

RESULTS

Grading of glomerular basement membrane thickness The results are shown in Table 1 The material consists of 111 controls (62 males, 49 females) and of 120 diabetic rats (66 males, 54 females) In the Table, the result of one biopsy from each rat is recorded In rats from which several biopsies were taken on different occasions, only the last biopsy is included Control animals of both sexes showed a clear increase of basement membrane thickness with age (Figs 2 and 3) The difference in thickness in rats aged 4 and 15 months was statistically significant for both sexes ($p<0\ 01$) This age-related in crease seemed to be more pronounced in males than in females At the age of 15 months the sex difference in basement mem brane thickness was almost significant ($p = 0\ 05$) There were no significant differences in thickness either between untreated and

TABLE 1 *Semiquantitatively Graded Glomerular Basement Membrane Thickness in Controls and Alloxan Diabetic Rats at Various Ages (cf Figs 2 3, 4 and 5)*

Thickness	Age in months									
	4		6		9		12		15	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
					Control rats					
4+										
3+									7	1
2+					5	4	8	1	15	10
1+	8	8	8	8	3	6	7	7	1	4
					Diabetic rats					
4+									1	
3+					1	1	5	2	17	20
2+			4	3	8	5	13	3	3	4
1+	8	8	4	4	2	3		1		

saline injected controls or between animals in which biopsy specimens had been taken once or on several occasions

In diabetic animals the same tendency to an increasing basement membrane thickness with age was observed The increase was, however, more marked in these animals than in the controls (Figs 4 and 5) There was no difference in the results obtained in controls and alloxan diabetic rats at the age of 4 months At 6 and 9 months of age, some of the diabetic animals had thicker basement membranes than the controls but the differences were not significant At 12 months of age, the difference was significant (males $p<0\,01$, females $p = 0\,02$) as well as at 15 months ($p<0\,01$ for both sexes) As regards basement membrane thickness, no significant differences between male and female diabetic rats at corresponding ages could be demonstrated or between animals which had undergone kidney biopsy once or on several occasions There was no correlation between graded basement membrane thickness and mean diuresis in 15 month old diabetic rats

In some rats both controls and diabetics, more than one kidney specimen (2–4) had been taken It was thus possible to follow the age-related increase in basement membrane thickness, and of other kidney changes, in individual cases These results were in good agreement with those presented in Table 1 which is based on the results of one biopsy from each rat

Quantitative determination of mesangial area As is evident from Table 2, there was an increase with age of the relative mesangial area in control animals The differences between the 4 and 15 months' values were significant ($p<0\,005$ for males $p<0\,001$ for females) When control and diabetic rats were compared, no differences were observed at 4 months of age At 15 months, however, the diabetic rats exhibited a significant increase in mesangial area compared with the controls ($p<0\,001$) No significant sex differences could be demonstrated Relating the mesangial values of 15 month old diabetic rats to their mean diuresis revealed no correlation

General description of other microscopic kidney changes In about 25 per cent of the older diabetic rats (12 and 15 months of age), a few glomeruli contained focal, intensely PAS positive, homogenous deposits resembling fibrinoid caps in human diabetic nephropathy (Fig 6) However no Kimmelstiel-Wilson nodules or hyaline arteriolosclerosis were seen Bowman s capsule was often somewhat thicker in the older rats (especially in diabetic animals)

The frequency and severity of tubulo interstitial kidney changes in rats aged 4 and 15

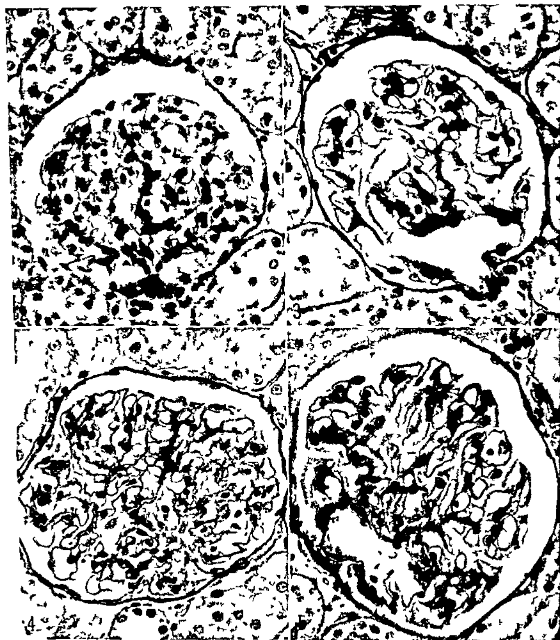


Fig 2 Glomerulus from a 4 month old male control rat The glomerular basement membrane thickness graded as 1 + PAS stain × 440

Fig 3 Glomerulus from a male control rat at 15 months of age showing basement membrane thickness of 2 + grade PAS sta n × 440

Fig 4 Glomerulus of a male rat d abetic for 12 months (15 months of age) The mesang al regions are prominent and the basement membrane thickness is graded as 3 + PAS stain × 440

Fig 5 Consp cuous basement membrane (the thickness graded as 4 +) and mesangial regions in a male rat with d abetes for 1? months PAS sta n × 440

TABLE 2 *Mean Relative Mesangial Area (Mesangial Area in per Cent of Tota Corpuscular Area within Bowman's Capsule) in Controls and Alloxan Diabetic Rats at 4 and 15 Months of Age*

Group	Age 4 months Mean ± SD	n	Age 15 months Mean ± SD	n
Control rats				
♂	8 1 ± 0 6	8	10 4 ± 1 7	10
♀	8 0 ± 0 6	8	10 5 ± 1 4	10
Diabetic rats				
♂	8 3 ± 1 2	8	13 2 ± 1 7	14
♀	8 1 ± 0 9	8	13 9 ± 1 9	24

SD – standard deviation, n – number of rats

TABLE 3 *Semiquantitatively Graded Tubular and Interstitial Changes in Controls and Alloxan Diabetic Rats at 4 and 15 Months of Age (cf Figs 7 and 8)*

Severity	Age 4 months Control rats ♂	♀	Diabetic rats ♂	♀	Age 15 months Control rats ♂	♀	Diabetic rats ♂	♀
3+							4	1
2+	1		1		7		8	3
1+	1	4	6	7	10	6	7	15
0	6	4	1	1	6	9	2	5

months are shown in Table 3 In 4 month old rats some focal changes of slight degree were often observed, especially in the diabetic animals These changes consisted mainly of tubular dilatation with flattened epithelium and PAS positive thickening of the tubular basement membrane (Fig 7) Except for nondiabetic females such tubular changes were more severe in the older animals particularly in diabetic males In addition to tubular lesions, older animals also exhibited focal interstitial changes such as cell infiltration mainly mononuclear cells and fibrosis Periglomerular fibrosis with hyalinized atrophic glomeruli was not an uncommon finding in old animals Colloid like tubular casts were also seen in cases with advanced tubulo interstitial lesions In such cases the histological picture resembled that of chronic pyelonephritis (Fig 8)

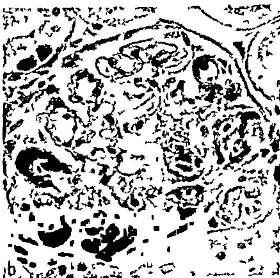


Fig 6 Focal, hyaline deposits resembling fibrinoid caps in a severely changed glomerulus of a male rat diabetic for 12 months PAS stain × 440

There were no obvious qualitative differences in tubulo-interstitial changes in non diabetic and diabetic animals The same applies to the aforementioned glomerular changes, except that focal PAS positive glomerular accumulations were seen only in diabetic rats



Fig 7 Focal tubular dilatation with epithelial flattening and thickening of the tubular basement membrane in a 4 month old rat diabetic for one month PAS stain × 250

Fig 8 Low power view of kidney of a rat diabetic for 12 months showing interstitial cell infiltration and fibros s tubular dilatation with colloid like casts and atrophic glomerulus PAS stain × 130

TABLE 4 *Serum Urea N (mg/100 ml) in Controls and Alloxan Diabetic Rats at Various Ages*

Group	Age 3 months		Age 3½ months		Age 15 months	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
Control rats			½ month after saline injection			
♂	17 6 ± 3 0	21	21 3 ± 3 2	14	22 0 ± 7 6	15
♀	17 2 ± 3 7	18	18 2 ± 5 0	11	20 9 ± 4 2	15
D abet c rats			½ month after alloxan inject on			
♂			35 2 ± 7 9	17	47 0 ± 12 1	10
♀			34 8 ± 6 7	14	34 1 ± 7 7	18

SD – standard deviation n = number of rats

Laboratory find ngs (Tables 4 and 5) There was a slight but significant increase in serum urea N in control animals aged from 3 to 15 months ($p<0\ 05$ for males $p<0\ 02$ for females) A corresponding comparison of serum creatinine values did not reveal any significant age related increase Two weeks after saline inject on control animals showed a tendency to a slight increase in urea N and a decrease in creatinine compared with the values at 3 months Diabetic rats exhibited a 100 per cent increase of urea N 2 weeks after induction of alloxan diabetes as compared with the controls at 3 months ($p<0\ 001$ for both sexes) *In the case of longer duration of* diabetes the mean value of urea N did not change in diabetic females In diabetic males however there was a significant increase of

TABLE 5 *Serum Creatinine (mg/100 ml) in Controls and Alloxan Diabetic Rats at Various Ages*

Group	Age 3 months Mean ± SD	n	Age 3½ months Mean ± SD	n	Age 15 months Mean ± SD	n
Control rats			½ month after saline injection			
♂	0 31 ± 0 07	19	0 23 ± 0 06	12	0 37 ± 0 18	10
♀	0 35 ± 0 08	16	0 30 ± 0 08	9	0 38 ± 0 11	11
Diabetic rats			½ month after alloxan injection			
♂			0 33 ± 0 12	17	0 46 ± 0 19	6
♀			0 31 + 0 20	10	0 34 ± 0 17	16

SD = standard deviation, n = number of rats

TABLE 6 *Urinary Albumin Content (mg/24h) in Controls and Alloxan Diabetic Male Rats at Various Ages*

Group	Age 3 months Mean ± SD	n	Age 3½ months Mean ± SD	n	Age 15 months Mean ± SD	n
Control rats			½ month after saline injection			
	4 1 ± 3 0	11	2 4 ± 1 1	11	29 3 ± 11 1	7
	(1 6 ± 1 3)		(1 1 ± 0 6)		(9 6 + 3 9)	
Diabetic rats			½ month after alloxan injection			
			0 9 ± 0 7	17	63 6 ± 37 9	7
			(0 4 ± 0 3)		(27 6 + 15 5)	

Numbers within brackets are albumin values adjusted to 100g body weight
SD – standard deviation, n number of rats

urea N in old rats as compared to young ones ($p<0\ 01$) Serum creatinine did not change significantly after alloxan injection or with the duration of diabetes

Urine albumin determinations were performed in some male rats (in female rats the amounts of albumin were often too small to be measured by the method used) The results appear in Table 6 There was a significant increase in urine albumin (mg/24 h) with age ($p < 0\ 001$ between 3 and 15 month values) Alloxan diabetic rats showed a significant decrease in albuminuria 2 weeks after alloxan administration compared with the values at 3 months ($p<0\ 01$) At 15 months of age, however, the diabetic rats had a significantly higher mean as compared to the controls ($p<0\ 05$ when using the original values, $p<0\ 02$ when urine albumin content was adjusted to body weight)

DISCUSSION

In the present study, glomerular basement membrane thickness has been graded in the light microscope on PAS stained sections The same mode of grading has been employed by *Säve Söderbergh et al* (31) and *Larsson* (18) on small skin vessels in human diabetics The basement membrane in glomeruli is however, much thinner than the walls of skin vessels but there was no difficulty in differentiating the thickness of the glomerular basement membrane into the 4 grades used in this study The expression "basement membrane thickness" is used here for practical

reasons in spite of the fact that the assessed PAS-positive structure of the glomerular capillary walls is not exactly defined anatomically However, some studies indicate that the PAS positive layer consists of lamina densa of the basement membrane (3, 23) Another question in this context is to what extent the intensity of the PAS stained structure influences the assessment of its thickness There is a possibility that a more intensely PAS stained structure is interpreted as being thicker than a less intensely stained one of the same thickness However, in this laboratory a fairly good correlation between light microscopically graded and electron microscopically measured glomerular basement membrane thickness has been found (11)

The second major light microscopic parameter used in the present study is the mesangial area The relative mesangial area as determined quantitatively in the light microscope has been reported to be increased in human diabetics as compared to that in normal subjects (15 34) The methods used in these studies were either point counting or planimetry It has been shown that the localization of the plane of section through the glomeruli is of little importance for the result, *i e* all sections except those close to the glomerular poles give essentially the same value of relative mesangial region (13) In the present work only glomerular sections with a chosen minimum size were used thus eliminating glomeruli sectioned near the poles

In addition to the glomerular lesions reported to occur in very old laboratory rats an age related thickening of glomerular basement membrane and an increase in mesangial area has been reported to occur in non diabetic rats during the first year (3 4 8) These findings were confirmed in the present study It is difficult to decide whether these glomerular changes should be considered as the result of physiological ageing phenomena alone or as ageing effects with some pathological processes superimposed Associated tubular lesions were also described (4 8) Dietary factors might influence the development of the glomerular and tubular changes (6, 17)

In diabetic animals with either spontaneous or experimental diabetes, glomerular changes which more or less resemble the glomerulosclerosis occurring in human diabetes have been reported (*cf* 7 25) The described major light microscopic glomerular lesions in alloxan diabetic rats consist of a progressive, PAS positive thickening of the capillary walls (or of their basement membrane) and/or of the mesangial regions (10, 14 21 22, 26) Glomerular changes usually develop in cases of untreated diabetes after 3–6 months (10, 14, 21, 22) This is in accordance with the results of the present study *Ørskov et al* (26) investigated insulin treated alloxan diabetic rats and observed no glomerular changes after diabetes of 5 months duration After 10 months however there were clear cut light microscopic glomerular changes in 75 per cent of the rats *Foglia et al* (9) induced diabetes in rats by pancreatectomy and found incipient glomerular lesions after 2 months diabetes Rats fed a high sucrose diet for periods of 9 to 18 months developed an impaired glucose tolerance but no manifest diabetes, and in several animals, thickening of the glomerular basement membrane and of the mesangial areas (29) The glomerular findings in starch fed controls were essentially normal

In almost all of the above mentioned studies as well as in the present study, localized PAS positive glomerular deposits have been found in the kidneys of diabetic rats The similarity between these accumulations and the exudative lesions seen in human diabetic nephropathy has been emphasized by some authors (24 29) However, no convincing Kimmelstiel Wilson nodules or PAS positive hyaline thickening of the arteriolar walls (outside the glomeruli) have been demonstrated in diabetic rats

It is reasonable to assume that the glomerular disease occurring in diabetic rats is secondary to the diabetic state since similar changes develop in the glomeruli irrespective of the method used for diabetes induction

It is not known whether or not the increased food intake may contribute to the development of the lesions in diabetic rats

Alloxan injection in rats damages not only the β cells of the pancreas but also the kidney tubules (33) Investigations by *Ørskov et al* (26) and *Vargas et al* (33) indicate that some of the persistent tubulo interstitial changes (apparently mainly tubular dilatation) seen in alloxan injected rats in which kidney protection has not been performed during alloxan administration are results of direct alloxan damage to the kidney In the present work, alloxan was administered under kidney protection Tubulo interstitial changes were seen not only in diabetic animals but also in nondiabetic controls The changes were of the same type in both groups but they were more common and more severe in the diabetic group Slight tubular lesions were often seen even in 4 month old rats, especially in the diabetic ones, *i e* before any glomerular disease could be demonstrated However there were some 15 month old diabetic rats with marked thickening of the glomerular basement membrane and mesangial regions that did not exhibit any clear cut tubulo interstitial lesions These results do not support the assumption that the tubulo interstitial lesions are caused by the glomerular disease (26) The genesis of the described tubular and interstitial lesions occurring in diabetic as well as in nondiabetic rats is unclear Similar changes in nondiabetic rats approximately one year old or older have been reported previously (2,5,8,28 32) *Berg* (4) described incipient lesions in 2 month old rats Different authors have different designations for the changes *Beverdge* & *Johnson* (5) classified them as chronic pyelonephritis In the present work the moderate and especially the severe lesions bore a resemblance to this disease

Ørskov et al (26) reported serum urea elevation in insulin treated alloxan diabetic rats after 5 and 10–11 months' diabetes It was stated that values of laboratory renal investigations on the days following alloxan administration under kidney protection were normal In the present work, a 100 per cent *increase of* serum urea *N* was found in diabetic animals 2 weeks after alloxan injection as compared with noninjected controls *With* longer diabetes duration (up to 12 months), the mean value was unchanged in females but significantly increased in male rats Serum creatinine did *not change significantly* after diabetes induction, but there was a tendency to increasing values with longer diabetes duration in males These findings indicate that the elevation of urea N seen after a short time in diabetic rats is not caused by renal insufficiency One of the factors known to influence serum urea concentration is the oral intake of protein (1) In diabetic rats, the food intake adjusted to body weight, was found to be increased up to 2–3 times that of the controls Thus increased protein intake might contribute to the elevated urea N of the diabetic animals in this study

An age related increase in urinary protein and albumin in laboratory rats have been reported (4, 27) In the present study, a similar tendency could be demonstrated with urine albumin content in male control rats Diabetic rats exhibited a marked decrease of albuminuria 2 weeks after alloxan injection The cause of this phenomenon is unknown Fifteen month old diabetic rats had a higher mean of urine albumin content than controls at the same age, but the inter individual variation was marked in the diabetic group

In summary, it has been shown in this study that not only alloxan diabetic but also nondiabetic rats develop age related microscopic changes in the kidneys The differences in the microscopical appearance in the two groups appear to be more quantitative than qualitative Thus in order to evaluate the main glomerular changes *i e* PAS positive thickening of the basement membrane and of mesangial area in alloxan diabetic rats it is important to attempt to quantitate these changes and compare the results with those from an adequate number of age matched controls

This work was supported by grants from the *Medical Faculty, University of Umeå*, the *Swedish Diabetes Association* and the *Nordic Insulin Fund*

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Acta path microbiol scand Section A 82, 211-219, 1974

GLOMERULAR BASEMENT MEMBRANE THICKENING IN RATS WITH LONG-TERM ALLOXAN DIABETES

A Quantitative Electron Microscopic Study

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Alloxan diabetes was induced in a total of 16 female rats Eight of them were untreated and 8 were given weekly injections of cyclophosphamide Five nondiabetic female rats served as controls Kidney biopsies were performed at 15 months of age, corresponding to a diabetes duration of 12 months Glomerular basement membrane thickness was measured on electron micrographs using two methods one described by *Jorgensen & Bentzon* (7) and the other by *Weibel & Knight* (22) (the latter method originally employed for measuring the alveolo capillary membrane in rat lung) The numerical agreement between the two methods was good The *Weibel* method however, showed definite theoretical and practical advantages A light microscopic grading of basement membrane thickness was fairly well correlated to the results of these electron microscopic methods The glomerular basement membrane was significantly thicker in the untreated diabetic rats than in the controls but there was no significant difference in thickness between the untreated and the cyclophosphamide treated diabetic group A positive correlation could be demonstrated between weighted diuresis and basement membrane thickness of the diabetic rats

A characteristic component of small vessel disease in human diabetes mellitus is the thickening of the capillary basement membrane (*cf* 8) In experimental diabetes only a few investigations have been published where basement membrane measurements have been performed Glomerular basement membrane thickness has been measured in alloxan diabetic rats (4 9) However the number of investigated rats is limited and the reported differences in thickness in control and diabetic animals do not seem to be firmly established

The standard method for measuring glomerular basement membrane thickness includes transmission electron microscopy and subsequent measurements of the peripheral basement membrane on micrographs (*cf* 15) Only such portions are selected for measurements where the plane of section appears to be perpendicular to the basement membrane, as judged by the distinctness of the adjacent plasma membranes However, apparent sharp plasma membranes do not guarantee that the basement membrane is cut in true cross section (15) Moreover, this method is exceedingly time consuming In 1964 *Weibel* &

Received 3 vii 73 Accepted 9 x 73

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Knight (22) described a method for measuring the thickness of the alveolo-capillary membrane in rat lung which was based on the conception that the characteristic thickness of that membrane with respect to gas diffusion is its harmonic mean thickness This method has apparently not been used previously for measuring glomerular basement membrane thickness

The object of the present study is to compare the glomerular basement membrane thickness of nondiabetic and alloxan diabetic rats A comparison of the thickness between untreated and cyclophosphamide treated diabetic rats is also included in order to test whether a reduction or inhibition of the glomerular basement membrane thickening could be induced in the treated diabetic rats Two ultrastructural procedures are used and the results are correlated with a light microscopic grading of the glomerular basement membrane thickness on paraffin sections stained with the periodic acid Schiff reagent (PAS) performed in a separate study (6)

MATERIAL AND METHODS

The basic experimental procedure was the same as that described previously (6) Twenty one female albino rats of highly inbred R strain were used The animals were kept in ordinary animal cages with free access to standard pellet food and water At the age of 3 months, 16 rats were starved for 48 hours and thereafter made diabetic with alloxan (55 mg/kg) administered intravenously under kidney protection (14) Eight of the diabetic rats were left untreated (no insulin was given) the rest were given weekly intraperitoneal injections of cyclophosphamide (Sendoxan®) 7 5–10 mg/kg from the third week after the alloxan injection to biopsy Five nondiabetic control animals were treated with saline instead of alloxan no cyclophosphamide was given to these animals The diabetic state was mainly controlled by monthly tests for glucosuria and measurements of 24 hour urine output adjusted to weight, as described elsewhere (6) Diabetic animals had a permanent diabetes with a mean diuresis of at least 20 ml/100 g/24 h, corresponding to a nonfasting blood glucose level of at least 200 mg/100 ml (6)

At the age of 15 months all rats underwent laparotomy in ether anaesthesia the pedicle of the right kidney was clamped-off and a superficial wedge shaped biopsy specimen was taken from the kidney cortex and processed for electron microscopy The tissue was cut into small blocks (0 5 × 0 5 mm) and fixed in cold 2 5 per cent phosphate buffered glutaraldehyde for 2 hours After rinsing in phosphate buffer the specimens were postfixed in cold 1 per cent phosphate buffered osmium tetroxide for 1 hour This was followed by dehydration in ethanol and embedding in Epon 812 (11) Thin sections were cut with an LKB Ultrotome at a feeding rate of 600–700 Å The sections were picked up on copper grids (LKB 300 mesh) and contrasted with uranyl acetate (21) and lead citrate (20) The sections were studied and photographed in a Zeiss EM 9 electron microscope (60 kV) The magnification was calibrated daily by means of a cross grating replica (Zeiss, Oberkochen West Germany)

Electron microscopic measuring of glomerular basement membrane thickness Two morphometric methods were employed described by *Jørgensen & Bentzon* (7) and by *Weibel & Knight* (22) and *Weibel* (23), respectively For the first method called the *Jørgensen* method in this study, 3 glomeruli from each of the 21 rats were used and the 63 glomeruli were coded Thirteen fourteen peripheral glomerular capillary loops from each glomerulus were selected and electron micrographs at about 8 800 × were made Measurements of basement membrane thickness were performed on prints at about 23 000 × and 10 capillary loops from each glomerulus were selected for that purpose Measurements were made only on peripheral portions of capillaries (mesangial portions of basement membrane were avoided) and only at points where both the endothelial and epithelial plasma membranes appeared sharp and where no round endothelial pores were seen In these regions the measurements were performed by planimetry of selected portions of basement membrane of a minimum length of 30 mm (range 30 to 180 mm) In the space between the epithelial foot processes and where pores interrupted the endothelial covering the planimeter followed the shortest distance between the points where the plasma membranes left their apparent attachments on the basement membrane Each portion of basement membrane was measured twice and the mean recorded as result The length of the portion was then measured twice by means of a chartometer and the mean was recorded The mean width of a selected basement membrane portion was calculated as the ratio between area and length In all 30 such measurements of each rat were performed The material was decoded when the measurements of all rats had been carried out and the results were analysed statistically Ten prints were selected at random and remeasured The coefficient of variation of the double measurements was 1 6 per cent

For morphometry according to *Weibel & Knight* (22) (the method here designated as the *Weibel*

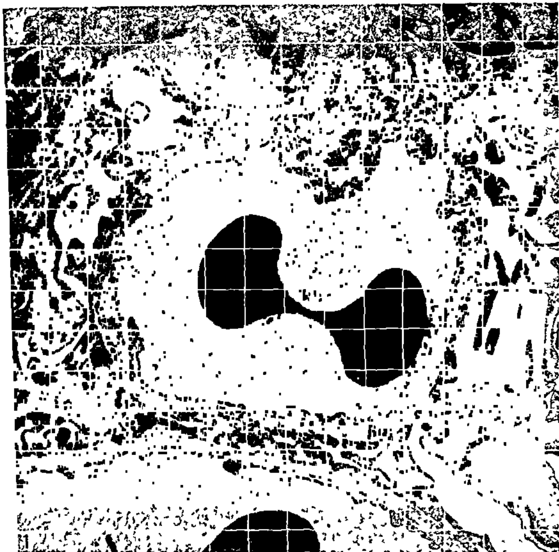


Fig 1 Electron micrograph showing a part of a glomerulus (from an untreated diabetic rat) A grid has been superimposed on the picture for measuring the harmonic mean thickness of the basement membrane according to the method of *Weibel & Knight (22)* Bm – Basement membrane, Ep = Epithelial cell, M Mesangium, Rbc = Red blood cell × 10,000

method), 5 glomeruli from each of 15 rats were used 5 nondiabetic controls 5 untreated and 5 cyclophosphamide treated diabetic animals (the 5 rats of each diabetic group were selected so that the degree of diabetes was similar between the groups) Three glomeruli from each rat were the same 3 glomeruli used in the *Jørgensen* method The 75 glomeruli were coded and electron micrographs were made at about 10,000 × (primary magnification about 1,750 ×) One picture was taken of each glomerulus the field of view was chosen at random, mainly according to the procedure of *Weibel* (23) To be accepted for measuring, each electron micrograph had to contain at least one peripheral capillary loop upon which at least 20 measurements could be made A transparent grid (23) was used for measurement on the pictures (fig 1), the distance between the lines was 10 mm Wherever a line, vertical or horizontal, crossed the basement membrane of peripheral loops (mesangial portions of basement membrane were avoided), the length of the line over the



Fig 2 Light micrograph of a glomerulus of a control rat Glomerular basement membrane thickness graded as 1 + PAS stain × 440

Fig 3 Light micrograph of a glomerulus from a diabetic rat Note the conspicuous capillary basement membrane (graded as 3 +) as compared with fig 2 PAS stain × 440

basement membrane (intercept length ι) was measured (in half mm s) In portions where the boundaries of the membrane were diffuse and indistinct, approximate measuring values were recorded The material was then decoded and the harmonic mean thickness of the basement membrane of each glomerulus (τ_h) was calculated according to the formula of *Weibel* & *Knight* (22)

$$\tau_h = 2/3 \quad \iota_h$$

where ι_h represents the harmonic mean of all the intercept lengths (ι) On each glomerulus 21–140 (mean 58) and on each rat 212–387 (mean 292) measurements were performed Since different numbers of measurements were made on different glomeruli and since interglomerular variation of basement membrane thickness in individual rats occurs the mean thickness for each rat was obtained by calculating the arithmetic mean of the harmonic mean thickness of each of the 5 measured glomeruli The variation coefficient calculated from double measurements of 10 prints was 3 9 per cent

Light microscopic grading of glomerular basement membrane thickness Kidney specimens from all rats at 15 months of age were fixed in cold ethanol and embedded in paraffin mainly according to *Sainte Marie* (18) Sections were cut at 5μ stained with PAS codified and studied at 400 × (objective 40 ×) Basement membrane thickness was assessed in 4 grades (1 + , 2 + , 3 + and 4 +) (figs 2 and 3) All glomeruli (about 40–80) of each section were examined and the average graded basement membrane thickness was recorded (6) It should be pointed out that the assessed PAS positive structure of the glomerular capillary walls is not exactly defined anatomically but some studies indicate that it consists of the lamina densa of the basement membrane (1, 12)

Statistical methods When the results of measured basement membrane thickness in the individual groups were compared Student s t test was used Correlation analyses were performed with *Pearson s* product moment correlation coefficient or *Spearman s* rank correlation coefficient (r_s)

RESULTS

The measuring results applying to each rat are shown in Table 1 Figs 4 and 5 provide examples of measured portions of basement membranes according to the *Jorgensen* method

As regards the measured basement membrane thickness there was in most cases good numerical agreement between the *Jorgensen* and the *Weibel* methods By comparison of the results of individual glomeruli a correla

tion coefficient of 0 70 ($p<0\,001$) was obtained A comparison of values of individual rats gave a correlation coefficient of 0 94 ($p<0\,001$)

By both methods, the basement membrane was about 40–50 per cent thicker in the untreated diabetic animals than in the non diabetic controls The difference between the groups was significant ($p<0\,001$) when comparing 5 control and 8 untreated diabetic rats as well as 5 animals of each group Some of the cyclophosphamide treated diabetic rats demonstrated slightly less basement membrane thickening than the untreated ones but statistical comparison of the untreated and treated group did not reveal any significant difference

The results of the light microscopic grading of glomerular basement membrane thickness on PAS stained sections are also found in Table 1 If compared with the values from the *Weibel* method, a correlation coefficient (r_s) of 0 76 ($p<0\,005$) was obtained A corresponding comparison with the values obtained by the *Jørgensen* method gave a correlation coefficient (r_s) of 0 79 ($p<0\,001$) when 5 rats were included and 0 60 ($p<0\,005$) when 8 rats were included in the diabetic groups

When the mean diuresis of each diabetic rat was related with the thickness of the basement membrane, a positive relationship was found by the *Weibel* method $r = 0\,76$ ($p<0\,02$), by the *Jørgensen* method $r - 0\,59$ ($0\,05<p<0\,10$) when 5 rats were analysed and $r = 0\,53$ ($p<0\,05$) when 8 rats were analysed in the groups

DISCUSSION

In the present study of measured glomerular basement membrane thickness in the rat there was a fairly close numerical agreement between the *Jørgensen* and the *Weibel* method As compared with the former method the *Weibel* method seems to be a more accurate one since the measurements are performed at random and the method does not include the uncertainty whether or not true cross sections of the basement membrane are measured Further advantages of the *Weibel* method are that the number of micrographs can be reduced and the measuring procedures are more rapid The fact that electron microscopic measurements and light microscopic assessment of glomerular basement membrane thickness showed a fairly *good correlation indicates that such a light* microscopic grading of PAS stained sections can serve as a rough indicator of glomerular basement membrane thickness in normal and in diabetic rats

Several authors have reported increased thickness of capillary basement membranes in human diabetes, including that of the glomerular tuft (*cf* 8) There are some reports on capillary basement membrane measurements in diabetic animals (2 3, 4 9, 10, 13, 16, 19), but a firmly established thickening of basement membranes in diabetic animals compared with nondiabetic ones of corresponding ages has only been demonstrated in a few studies using diabetic dogs (2, 16)

Concerning animals with experimental diabetes, *Bloodworth et al* (2) reported increased thickness of glomerular, retinal and muscle capillary basement membranes in alloxan diabetic and metasomatotrophin diabetic dogs as compared with controls *Lazarow* (9) has given a short report on measurements of glomerular basement membrane thickness in alloxan diabetic rats This author compared 4 rats diabetic for 7 months and 3 age matched controls and found a 19 per cent increase of basement membrane thickness in the diabetic group According to *Lazarow*, the difference between the groups was highly significant ($p<0\,001$) However, using the t test where n = the number of investigated *animals (and not the number of measured* glomeruli) the difference is not significant at the 5 per cent level ($0\,05<p<0\,10$) *Ditscherlein et al* (4 5) also reported a measured thickening of the glomerular basement membrane in untreated alloxan diabetic rats They investigated rats at different ages but the number of animals studied on each occasion was so small (2–4 diabetic and 1 2 con


Rh
En
Bm
Ep
Fp
Mc
5

TABLE 1 *Results of Electron Microscopic Measurements and Light Microscopic Grading of Glomerular Basement Membrane Thickness in 3 Groups of 15 Month Old Female Rats*

Rat no	Electron microscopically measured mean thickness (Å) *Jorgensen* method	*Weibel* method	Light microscopically graded thickness (1+, 2+, 3+, 4+)	Mean diuresis ml/100g/24h during the period 3–15 months of age
I Nondiabetic control rats				
1	1950	2230	2	
2	1960	2380	2	
3	2300	2410	2	
4	2350	2290	2	
5	2170	2420	1	
Mean ± SD	2150 ± 190	2350 ± 80		
II Untreated diabetic rats				
6	2930		2	67
7	3510		2	68
8	3220		3	48
9	3180	3460	3	82
10	3180	3090	3	35
11	3060	3270	3	41
12	3020	3060	3	44
13	3600	3320	3	79
Mean ± SD	3210 ± 230 (3210 ± 230)	3240 ± 170		58 ± 18 (56 ± 22)
III Cyclophosphamide treated diabetic rats				
14	3380		3	66
15	3400		3	72
16	3410		2	58
17	3090	3320	3	78
18	2700	2980	1	44
19	2550	2610	1	21
20	3160	3210	3	82
21	2770	3010	3	67
Mean ± SD	3060 ± 340 (2850 ± 260)	3030 ± 270		61 ± 20 (58 ± 26)

Numbers within brackets are the mean values ± SD of the last 5 rats of the group
SD = standard deviation

Fig 4 Electron micrograph of a capillary loop in a glomerulus of a control rat The part of basement membrane between the solid black lines was measured by the method of *Jorgensen & Bentzon* (7) Note the normal distribution of the epithelial cell foot processes. Bm = Basement membrane, Ep = Epithelial cell Fp = Foot processes, Rbc = Red blood cell × 26,000

Fig 5 Electron micrograph of a glomerular capillary loop from a diabetic rat The measured portion of basement membrane (according to the method of *Jorgensen & Bentzon* (7)) is delimited by solid black lines Round endothelial pores are clearly visible Note the fusion of the foot processes Bm = Basement membrane, Cl = Capillary lumen, En = Endothelial cell, Ep = Epithelial cell, Fp = Foot processes, M = Mesangium, P = Endothelial porus. × 26,000

trol rats) that no definite conclusions can be drawn concerning differences of basement membrane thickness between diabetic and nondiabetic rats Measurements of glomerular basement membrane thickness have also been performed on selected rats developing *impaired glucose tolerance* (but no manifest diabetes) and light microscopic glomerular changes during long-term (12–18 months) sucrose feeding (17) Compared with starch fed controls, the sucrose fed animals developed significant basement membrane thickening

In the present investigation, a 40–50 per cent increase of glomerular basement membrane thickness in alloxan diabetic rats with 12 months diabetes compared with age-*matched controls was demonstrated It can* thus be stated that alloxan diabetic rats develop a definite thickening of the glomerular basement membrane as do human subjects with long-term diabetes (*cf* 8) In the present study, a positive relationship between the measured basement membrane thickness and the mean weight adjusted diuresis of diabetic rats could also be demonstrated

*A therapeutical trial with cyclo*phosphamide is also presented Whereas some of the cyclophosphamide treated rats developed a reduced basement membrane thickening as compared with untreated animals, no significant difference between the groups could be demonstrated A more detailed study of the effect of cyclophosphamide treatment *will* be *published elsewhere*

The author is indebted to Prof *Herbert Helander*, Institute of Anatomy, University of Umeå, for valuable help concerning the *Weibel* method

This work was supported by grants from the *Medical Faculty, University of Umeå*, the *Swedish Diabetes Association* and the *Nordic Insulin Fund*

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Acta path. microbiol scand Section A 82, 220-227, 1974

OCCURRENCE OF IMMUNOGLOBULIN AND COMPLEMENT IN THE GLOMERULI OF RATS WITH LONG-TERM ALLOXAN DIABETES

An Immunofluorescence Study

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The occurrence of immunoglobulin G (IgG) and complement (β_{1C}) in the kidneys of untreated alloxan diabetic rats has been investigated by immunofluorescent technique using ethanol fixed, paraffin embedded tissue The results were compared with light microscopical glomerular changes described previously IgG could be demonstrated in glomeruli (mainly in the mesangium) of almost all diabetic rats as early as one month after diabetes induction i e before light microscopical glomerular lesions were present The frequency and severity of IgG deposition did not change with increasing diabetes duration up to 12 months. There was a positive correlation between the amount of mesangial IgG and the severity of the diabetic state as measured by diuresis Slight amounts of mesangial IgG were also found in a few old nondiabetic controls. β_{1C} could not be demonstrated with certainty in glomeruli when using the paraffin embedding method However the use of freezing technique in a few diabetic rats permitted detection of β_{1C} in the mesangium of some cases

The pathogenesis of microangiopathy in diabetes mellitus is still unsettled In human diabetics, plasma proteins, and in particular immunoglobulins, have been demonstrated in increased amounts in the walls of small vessels, including the glomeruli (*cf* 20) The presence of immunoglobulins has raised the question whether immunological mechanisms play a pathogenetic role in the development of diabetic microvascular disease (*cf* 20)

In attempts to elucidate this question, studies of animals with diabetes might be valuable In a preliminary communication 1969, the present author reported deposition of gammaglobulin with immunofluorescent technique in the glomeruli of alloxan diabetic rats (5) Later, *Mauer et al* (11, 13) presented a detailed immunofluorescent study concerning the occurrence of glomerular immunoglobulin G (IgG) and complement (β_{1C}) in alloxan diabetic rats These authors reported that rats diabetic for 2 to 12 months exhibited increasing amounts of these proteins mainly in the mesangium In some rats diabetic for 16 months there was a linear glomerular basement membrane distribution

Received 3 vii 73 Accepted 9 x 73

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of IgG and β_{1C} Immunoglobulin and complement deposition appeared to antedate light microscopic glomerular changes Such immunohistochemical reports on diabetic animals are otherwise few in number Gammaglobulin and β_{1C} have been demonstrated in glomeruli of KK mice with spontaneous hereditary diabetes (19)

The present study is an extension of the preliminary findings reported in 1969 The occurrence of rat IgG and β_{1C} has been investigated in alloxan diabetic rats and the results are correlated with the appearance of light microscopic kidney changes These results will be referred to in other studies from this laboratory where different therapeutical regimens have been given to alloxan diabetic rats

MATERIAL AND METHODS

Albino rats of both sexes of highly inbred R strain were used (6) They were given standard food and water *ad libitum* Diabetes was induced at the age of 3 months with alloxan (55 mg/kg), administered intravenously during kidney protection (15) after 48 hours of starvation No insulin was given The diabetic state was mainly controlled by monthly tests for glucosuria and measurements of 24 hour urinary output adjusted to weight as described elsewhere (6) All diabetic animals (35 males and 38 females) had a permanent diabetes, and each rat had a mean diuresis of at least 20 ml/100 g/24 h, which corresponded to a non fasting blood sugar level of at least 200 mg/100 ml (6) Control rats (35 males and 33 females) were either left untreated (30 rats) or given a saline injection (38 rats) instead of alloxan at 3 months of age

At the ages of 4 6, 9 and 15 months (corresponding to a diabetes duration of 1, 3, 6 and 12 months respectively), a number of control and diabetic animals were biopsied and sacrificed Laparotomy was performed under ether anaesthesia the pedicle of the right kidney was clamped off and a biopsy specimen for ordinary light microscopy was taken from that kidney The right renal artery and the upper part of abdominal aorta was ligated and a cannula inserted into the aorta below the renal arteries An infusion set with a bottle containing saline was connected to the cannula. The pressure of the infusion bottle was adjusted to 120 mm Hg and the left kidney was perfused with about 100 ml of saline Macro- and microscopically, the kidney appeared to be blood free after that procedure A wedge shaped piece of tissue was taken from the perfused kidney

Both biopsy specimens were processed according to the method of *Sainte-Marie* (17) with a slight modification, *i e* fixed in cold 95 per cent ethanol for about 24 hours, dehydrated in cold 99 per cent ethanol for 3 × 1 hours, cleared in methyl benzoate (for about 24 hours) and in xylene for 3 × 5 minutes The tissue was then placed in a paraffin oven at about 58° C overnight Blocks were stored in refrigerator until used

Specimens from perfused kidneys were sectioned at 5 μ The sections were picked up on glass slides, deparaffinized in xylene for 3 × 5 minutes, passed through graded ethanol and finally washed in phosphate buffered saline pH 7 2 for 3 × 5 minutes Indirect immunofluorescence staining was used After every application of antiserum the slides were incubated in a moist chamber for 30 minutes and then washed in buffered saline for 3 × 5 minutes Finally, they were covered with cover glass over a drop of mounting medium consisting of a solution of polyvinyl alcohol and glycerol in buffered saline pH 7 2 (16)

Rabbit serum against rat IgG was prepared in the following manner IgG was isolated from pooled rat serum by double elution from a diethyl aminoethyl-cellulose column* The IgG solution was mixed with Freund s complete adjuvant (Difco Laboratories Detroit, Michigan) and injected subcutaneously in rabbits on a single occasion After 3 weeks, the animals were bled by heart puncture and the rabbit serum was tested against rat serum in immunoelectrophoresis§ Rabbit sera that produced a single precipitation line, representing rat IgG, were used Rat β_{1C} (C 3) was prepared from fresh pooled rat serum according to the method of *Mardiney & Muller Eberhard* (10) The zymosan complement complex was suspended in incomplete Freund's adjuvant (Difco Laboratories) Rabbits were immunized with a single injection and antisera were collected after 3 weeks The antisera were mixed with rat IgG and immunoelectrophoresis was performed after centrifugation§ Antisera that produced a single precipitation line in the β globulin region were used The fluorescein conjugated antiserum for the second staining step was a commercial sheep anti rabbit immunoglobulin (Statens Bakteriologiska Laboratorium, Stockholm) with a 2 7 molar F/P ratio and a 10 fold dilution was found to be suitable

The following control stainings were performed

1) The sections were only covered with the conjugated antiserum

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* Kindly performed by dr *R Lorentzon*, Department of Clinical Chemistry, University Hospital, Umeå.

§ Kindly performed by dr *O Rudolphi*, Department of Clinical Chemistry, University Hospital, Umeå.



Fig 1 Glomerulus from a male control rat at 15 months of age The capillary basement membrane thickness graded as 2+ PAS stain × 440

Fig 2 Glomerulus of a male rat diabetic for 12 months (15 months of age) Note the conspicuous capillary basement membrane (the thickness graded as 3+) and mesangial regions as compared with Fig 1 PAS stain × 440

2) The sections were covered with normal rabbit serum and then, after rinsing, the conjugated antiserum was added
3) Coincidental application on the sections of rat IgG and rabbit anti rat IgG serum After washing, the conjugated antiserum was added

All of these control stained sections were negative or showed a marked diminution of fluorescence compared to ordinary stained sections

In order to test the tissue preparation and antisera for demonstrating rat IgG and β_{1C}, Masugi-nephritis was induced in some rats (*cf* 1) Kidneys from normal rats were homogenized, washed with saline and mixed with Freund's complete adjuvant (Difco Laboratories) Rabbits were immunized by subcutaneous injections of the suspension once a week during 6 weeks Rabbit sera were collected 10 days after the last injection and given intravenously to young normal rats These were sacrificed about 10 days after serum injection The left kidney was perfused with saline and processed according to *Sainte Marie* (17) as described above Kidney sections from one rat were stained with the antisera for demonstrating rat IgG and β_{1C} A strong, specific, continuous fluorescence for both proteins was seen along the glomerular capillary walls, thus indicating that the tissue preparation and the antisera used worked satisfactorily

All sections were stained on the same occasion with the same batch of antisera for demonstrating IgG and β_{1C} The slides were coded and examined within a few days after staining in a Zeiss fluorescence microscope having a 200 W HBO high tension mercury vapour bulb as the light source The exciter filter was BG 3 and barrier filter No 50 or 53 Photographs were taken with an Agfapan 400 film The fluorescence (mainly its intensity) was arbitrarily graded semiquantitatively as negative, 1+, 2+ and 3+ Each section contained about 40–80 glomeruli

In 6 diabetic rats, kidney biopsy specimens (both from perfused and non perfused kidneys) were also quick frozen in isopentane precooled in liquid nitrogen and stored at −70° C until used The frozen tissue was sectioned in a cryostat microtome and stained with antisera as described above

Paraffin embedded tissue from the non perfused kidney cut at 5 μ was stained with periodic acid Schiff (PAS) and van Gieson's method (Figs 1 and 2) The glomerular basement membrane thickness was graded semiquantitatively as described elsewhere (6) Relative mesangial area (mesangial area in per cent of total corpuscular area within Bowman's capsule) was determined by point counting method on PAS stained sections cut at 3 μ (6)

When comparing the results of graded fluorescence between groups, the non parametric rank sum test of *Wilcoxon* was used $p < 0.05$ was

chosen as the level for statistical significance Correlation analyses were performed with *Spearman's* rank correlation coefficient (r_s)

TABLE 1 *Semiquantitatively Assessed Specific Fluorescence for IgG in the Mesangium of Controls and Alloxan Diabetic Rats at Various Ages Saline Perfused Paraffin Embedded Kidneys Used*

Amount of IgG	Age in months 4 ♂	4 ♀	6 ♂	6 ♀	9 ♂	9 ♀	15 ♂	15 ♀
CONTROL RATS								
3+								
2+								
1+							5	2
0	5	5	6	8	4	6	15	12
DIABETIC RATS								
3+		3	2	3	1	1	1	5
2+	4	2	5	3	2	4	7	6
1+	2	3		1	3	1	2	4
0	2				1	1	3	1

RESULTS

The results concerning IgG localization in glomeruli of control and diabetic rats of both sexes are shown in Table 1 When glomerular fluorescence occurred, it was regularly localized in the mesangium (Figs 3, 4 and 5) In diabetic animals a concomitant faint fluorescence was sometimes seen along some of the glomerular capillary walls in a segmental linear pattern In exceptional cases, a weak fluorescence could be demonstrated along Bowman's capsule No fluorescence could be detected in vessel walls outside the glomeruli In some rats (diabetic and nondiabetic), a slight fluorescence for IgG was seen in the interstitial connective tissue Mononuclear cells with cytoplasmic fluorescence were a common finding in this tissue both in control and diabetic animals (in about two thirds of the rats of both groups) with a tendency of increasing frequency with age The cytoplasmic IgG might represent antibodies produced by the cells

As is evident from the Table, mesangial IgG could be demonstrated in diabetic rats as early as one month after alloxan injection The frequency and quantity of mesangial IgG did not show any significant changes with longer diabetes duration Diabetic females appeared to have somewhat more of IgG than corresponding males but the differences were not significant With the exception of a slight amount in some 15 months old animals, control rats did not exhibit glomerular fluorescence The difference of mesangial fluorescence between control and diabetic animals was significant at all corresponding ages (p ranging between < 0.01 and < 0.05) When relating the amount of mesangial IgG of diabetic rats with their mean diuresis, a positive correlation was found ($r_s = 0.66, p < 0.001$)

Specific fluorescence for β_{1C} was not observed with certainty in any of the investigated glomeruli of the present experimental series Staining of frozen unfixed renal tissue from 6 diabetic rats (4 and 9 months of age) showed however, in 3 cases (both in the perfused and non perfused kidney) a definite fluorescence for β_{1C} in the mesangium (Fig 6) A comparison of the results of staining for IgG between frozen and ethanol fixed paraffin embedded specimens from 6 diabetic animals was also performed There was usually a more intense fluorescence in the mesangium of the unfixed material but, on the other hand this method exhibited a markedly inferior tissue preservation as compared with the *Sainte Marie* method (17) The localization and intensity of glomerular fluorescence were both about the same in non perfused and perfused kidney specimens when freezing technique was employed Mesangial IgG was demonstrated with this method in 2 investigated diabetic animals aged 4 months (one month's diabetes)

DISCUSSION

In this study the kidney specimens were fixed in cold ethanol and embedded in paraffin according to *Sainte Marie* (17) This method was chosen for immunofluorescence studies because it is more convenient to use and gives a better tissue preservation than the









Fig 3 Glomerulus of a female rat with diabetes for 12 months with IgG of 3+ intensity in the mesangium Fluorescence also in some capillary walls × 330

Fig 4 Glomerulus of a male rat diabetic for 12 months. Moderate staining intensity (2+) for IgG in the mesangium and in an occasional capillary loop Some autofluorescence of tubular cells × 330

Fig 5. Glomerulus of a female rat diabetic for one month There is mesangial staining for IgG of 2+ intensity × 330

Fig 6 Frozen section of kidney of a female rat diabetic for one month, showing bright mesangial fluorescence for β_{1C} × 330

freezing method (7, 8, 17) *Lindquist et al* (9) used the *Sainte Marie* method for the demonstration of IgG and β_{1C} in transplanted rat kidneys In the present investigation some biopsy specimens from diabetic rats were also frozen and sectioned on a cryostat microtome for comparison Glomerular fluorescence for IgG was usually somewhat stronger using the latter method

β_{1C} could not be demonstrated with certainty in glomeruli of ethanol fixed, paraffin embedded tissue, but in 3 of 6 frozen specimens from diabetic rats a clear, mesangial fluorescence for β_{1C} was seen (2 of the rats with strong fluorescence had a diabetes duration of one month) The failure of the *Sainte Marie* method to demonstrate glomerular β_{1C} in diabetic animals may be due to changes of the antigenic sites of the complement induced by fixatives and by heat (paraffinization at about 58° C) On the other hand, a strong glomerular fluorescence for β_{1C} was seen in sections of a perfused Masugi nephritic rat kidney processed according to *Sainte Marie* A possible explanation of this discrepancy might be that the nephritic kidney contains larger amounts of β_{1C} than that of a diabetic rat

At the beginning of the present investigation, sections from non perfused kidneys were used for immunofluorescence studies A short communication of preliminary results on non perfused kidneys was published 1969 (5) as mentioned above There was, however a problem with fluorescence of remaining blood in the glomerular capillary lumina in these kidneys which interfered with the assessment of fluorescence in the capillary walls and in the mesangium Therefore, saline perfusion of the kidneys was started which made the glomeruli practically free from blood and easier to assess as regards tissue fluorescence

The results of the present investigation with perfused kidneys clearly show that untreated alloxan diabetic rats have an increased incidence and quantity of IgG in the glomeruli mainly in the mesangium as compared with nondiabetic controls Almost 90 per cent of the diabetic rats exhibited glomerular IgG as early as one month after alloxan injection The frequency and severity of IgG deposition did not show any significant tendency to increase with the diabetes duration There was, however, a positive relationship between the amount of mesangial IgG and the severity of the diabetic state, as measured by urine output, in individual rats

In a previous report (6), light microscopic kidney changes in the rats used in the present investigation were studied No certain glomerular lesions could be demonstrated in diabetic rats compared with controls at the age of 4 months (one month of diabetes) At 6 and 9 months of age, some of the diabetic animals had increased glomerular basement membrane thickness as compared with control animals but the differences were not significant However, when 15 month-old animals were compared the diabetic rats showed a significant increase of graded basement membrane thickness as well as measured mesangial area Thus it appears that glomerular IgG deposition precedes light microscopic glomerular changes in the diabetic rats No significant correlation could be demonstrated when the occurrence of mesangial IgG was related to the graded glomerular basement membrane thickness and to the determined mesangial area in 15 month old diabetic rats

A comparison of the present results concerning glomerular IgG with those reported by *Mauer et al* (13) shows certain differences. Using rank sum test on their material there is a significant ($p < 0.01$) increase of the frequency and amount of that protein in the glomeruli from 2 to 4 months' diabetes However, when excluding the 2 month values no significant change of glomerular IgG with the diabetes duration can be demonstrated Thus, the only definite discrepancy between the present study and that of *Mauer et al* concerning glomerular IgG appears to be the amount of that protein demonstrated during the early phase of alloxan diabetes Besides different rat strains, there are some technical differences between the two investigations concerned In the present

report, alloxan was given during kidney protection, the kidneys were perfused with saline before biopsy, the tissues were ethanol fixed and paraffin embedded and an indirect staining method was used to demonstrate IgG *Mauer et al* employed unfixed frozen specimens and a direct immunofluorescent technique It is possible that the mentioned discrepancy of glomerular IgG between the present study and that of *Mauer et al* might be explained by different methodological sensitivity in demonstrating that protein In both studies mesangial IgG deposition appeared to precede light microscopic glomerular changes

When comparing the results of glomerular IgG localization in alloxan diabetic rats and in human diabetics there is a definite difference in alloxan diabetic rats the main localization is in the mesangium in man it resides in the capillary walls and not in the mesangium with the exception of Kimmelstiel Wilson nodules (3, 20) Whether this discrepancy of IgG localization signifies a fundamental difference as regards the development of diabetic glomerular lesions, cannot be answered at present Mesangial localization might occur at an earlier stage of glomerular disease than localization in the capillary walls (13)

It is not known at what stage of diabetic kidney disease immunoglobulins appear in the glomeruli Thus it is an open question whether they are deposited before or after other biochemical, structural or physical glomerular alterations have occurred In any event immunoglobulin deposition in diabetic rats seems to precede detection of glomerular lesions by ordinary light microscopy Immunoglobulins have been reported to occur in glomeruli in a few investigated cases of human diabetics where no or only minimal glomerular changes could be detected in ordinary light microscopy (20)

Furthermore, it is still an unsettled question what mechanism (or mechanisms) underlies glomerular immunoglobulin deposition It might be due to immunological reactions Eluates from diabetic kidneys containing glomerular IgG did not, however react with normal glomerular basement membrane (4, 20) Nor does the homogenous linear pattern of immunoglobulin localization in the glomeruli of human diabetics resemble the picture seen in immune complex glomerular diseases (14) In addition demonstration of complement in glomerular capillary walls in diabetic subjects has often failed (3 4, 14) As a consequence non immunological binding of proteins has been considered *e g* trapping of proteins in damaged vessel walls and altered vascular permeability to proteins (2) Evidence of increased capillary permeability has been presented by *Scher & Lawrence* (18) in alloxan diabetic rats 4–6 weeks after alloxan injection Altered mesangial function in diabetics has also been proposed (20) It has been shown that rats made nephrotic with aminonucleoside of puromycin exhibit increased mesangial uptake of circulating macromolecules (12)

The consequences of glomerular immunoglobulin accumulation for the development of diabetic glomerulosclerosis are also unknown In the present work with diabetic rats there was a discrepancy between the stationary occurrence of glomerular IgG and the progressive increase of mesangial area and of basement membrane thickness with diabetes duration Furthermore there was no significant relationship between the severity of the latter changes and the amount of mesangial IgG in rats diabetic for 12 months

In an attempt to elucidate the above mentioned questions, the influence of insulin and cyclophosphamide treatment as well as neonatal thymectomy, on IgG deposition and other morphological glomerular changes in alloxan diabetic rats are at present being studied in this laboratory

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This work was supported by grants from the *Medical Faculty University of Umeå* the *Swedish Diabetes Association* and the *Nordic Insulin Fund*

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Acta path microbiol scand Section A 82, 228–234, 1974

INFLUENCE OF INSULIN TREATMENT ON GLOMERULAR CHANGES IN RATS WITH LONG-TERM ALLOXAN DIABETES

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The hypothesis that insulin itself participates in the pathogenesis of diabetic microangiopathy was tested in alloxan diabetic male rats by giving them daily injections of a commercial protamine zinc insulin for 3 months after 9 months of untreated diabetes Insulin doses were adjusted so that the urine volumes and glucosuria were kept at low levels When compared with untreated alloxan diabetic rats of the same diabetes duration (12 months), the insulin treated animals showed a significantly decreased incidence and quantity of mesangial IgG There was also a tendency to less glomerular basement membrane thickness and mesangial area in the treated rats, but the differences between the two groups were not significant The glomeruli of nondiabetic insulin treated animals did not differ from those of untreated controls These results do not support the assumption that the presence of insulin contributes to the pathogenesis of diabetic glomerulosclerosis The findings favour the concept that glomerular disease in diabetes is the result of insulin deficiency Furthermore, deposition of glomerular IgG in alloxan diabetic rats is evidently a reversible phenomenon

Insulin has been suggested to be actively involved in the pathogenesis of diabetic microangiopathy (1, 2, 5, 8, 10, 14, 23, 32, 35) It has, for instance, been claimed that vascular disease might be the result of immunological reactions with circulating insulin antigen antibody complexes (14) It has also been proposed that complement fixing insulin-immunoglobulin G (IgG) complexes, in which IgG does not constitute antibodies to insulin, might be formed in diabetics and cause vascular damage (8)

The present study was undertaken to test the hypothesis that insulin takes part in the development of the diabetic microangiopathy Rats with long term alloxan diabetes were used These animals have an insulin deficiency type of diabetes (24) and they develop glomerular changes resembling diffuse glomerulosclerosis in human diabetes (17 18), including deposition of glomerular IgG (19, 22) The effect of insulin treatment on the mentioned renal changes in the diabetic rats was investigated Comparative studies of glomerular structure in untreated and insulin treated diabetic animals are few in number, and those available are lacking in detailed presentation of the results Moreover, they do not include quantitative microscopical methods, nor immunofluorescent investigations concerning immunoglobulin content in the glomeruli

Received 12 x 73 Accepted 12 x 73

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MATERIAL AND METHODS

The details of the basic experimental procedure have been described previously (17) Male albino rats of highly inbred R strain were used The animals were fed standard pellet food and supplied with tap water *ad libitum*

Diabetes was induced at the age of 3 months by an intravenous alloxan injection (55 mg/kg) under kidney protection (17) The diabetic state was mainly controlled by monthly tests for glucosuria (Clinistix®, Ames Company) and of 24 hour urine measurements adjusted to weight (17) Nonfasting blood glucose content and quantitative urine glucose values were usually determined at 2 weeks as well as 6 and 12 months after alloxan injection Only animals with a persistent diabetes with a mean weighted diuresis of at least 20 ml/100 g/24 h, corresponding to a blood glucose level of at least 200 mg/100 ml (17), were used Nondiabetic control rats were either left untreated or they were injected with saline at 3 months of age

At the age of 12 months, 19 nondiabetic control and 17 diabetic rats were treated with one daily subcutaneous injection, some control rats only 6 days a week, of a commercial protamine zinc insulin (mixture of bovine and porcine insulin), 40 U/ml (Vitrum, Stockholm) for 3 months, *i e* from 12 to 15 months of age The remainder of the animals were untreated The degree of diabetes, as measured by diuresis, from alloxan injection up to 12 months of age did not differ significantly between the untreated and the insulin treated diabetic group, the 24 hour urine output adjusted to weight being 47 ml/100 g/24 h $\pm$ 11 (SD) (n = 21) and 56 ml/100 g/24 h $\pm$ 17 (SD) (n = 12), respectively The mean daily dose of insulin for the control rats was 1 41 U/100 g $\pm$ 0 11 (SD) and for the diabetic rats 1 21 U/100 g $\pm$ 0 25 (SD) In the diabetic rats, insulin doses were calculated on the basis of daily urinary output and glucosuria Consequently, these animals had to be kept in metabolic cages during the treatment period The mean 24 hour urine output of the diabetic rats during insulin therapy was 6 5 ml/100 g $\pm$ 1 0 (SD) Blood sugar concentration and quantitative urine sugar values were measured at least once during insulin treatment, and always during the days before the final biopsy In insulin treated diabetic animals, the blood glucose level was found to fluctuate extensively during the day 5–10 hours after insulin injection the values were usually 25–150 mg/100 ml and 20 24 hours after injection usually 150–300 mg/100 ml Quantitative urine sugar was always less than 0 35 g/100 g/24 h, whereas untreated diabetic rats had more than 2 g/100 g/24 h Insulin treated control rats had normal blood sugar levels, always less than 120 mg/100 ml 24 hours after insulin injection, and no glucosuria Of the insulin injected animals, 12 diabetic and 11 nondiabetic rats survived the 3-month treatment period The most frequent cause of death among the treated rats was apparently hypoglycemia The mean body weight of the untreated diabetic rats at the end of the experiment was 232 g $\pm$ 43 (SD) and that of the insulin treated diabetic rats 273 g $\pm$ 26 (SD) This difference is significant ($p < 0 01$) A corresponding comparison of body weight between the two nondiabetic groups did not reveal any significant difference

At 15 months of age the rats underwent laparotomy for kidney biopsy before they were sacrificed Specimens, almost always from the right kidney, were regularly taken for ordinary light microscopy (17) In some animals, additional small specimens were taken from the right kidney and processed for transmission electron microscopy (18) In all the insulin treated rats, both diabetic and nondiabetic, and in most of the other animals, the left kidney was perfused *in situ* with saline through the renal artery just after the rat had been sacrificed by bleeding Tissue from this kidney was embedded mainly according to *Sainte Marie* (28) for immunofluorescence staining, as described previously (19)

Light microscopic investigations The sections were stained by the periodic acid Schiff (PAS) and van Gieson techniques On coded PAS stained sections the average thickness of the PAS positive glomerular basement membrane of each specimen was semiquantitatively graded, using a 4 degree arbitrary scale (17) The relative mesangial area, *i e* mesangial area in per cent of total corpuscular area within Bowman's capsule, was estimated by the point-counting method (17)

Immunofluorescent studies Sections, about 5 μ thick, from perfused paraffin embedded kidney specimens, were processed for immunofluorescent staining (19) An indirect technique was used both for demonstrating IgG and β_{1C} . The first layer consisted of a rabbit serum against rat IgG and rat β_{1C} , respectively Both antisera were prepared in this laboratory (19) A fluorescein tagged sheep anti rabbit immunoglobulin (Statens Bakteriologiska Laboratorium, Stockholm) served as the second layer The appropriate control stainings, performed as described previously (19), were consistently negative for specific fluorescence All sections were stained, coded and examined in a Zeiss fluorescence microscope at one and the same time Grading of glomerular fluorescence (mainly its intensity) was made using an arbitrary semiquantitative 4 point scale (negative, 1+, 2+ and 3+) (19)

Electron microscopic measurements Epon embedded kidney tissue from the following 3 groups of rats were used, *viz*, 4 nondiabetic animals, not treated with insulin, 5 untreated and 5 insulin treated diabetic rats The diabetic rats were selected so that the degree of diabetes, as measured

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The present study was undertaken to test the hypothesis that insulin takes part in the development of the diabetic microangiopathy Rats with long-term alloxan diabetes were used These animals have an insulin deficiency type of diabetes (24) and they develop glomerular changes resembling diffuse glomerulosclerosis in human diabetes (17-18), including deposition of glomerular IgG (19, 22) The effect of insulin treatment on the mentioned renal changes in the diabetic rats was investigated Comparative studies of glomerular structure in untreated and insulin treated diabetic animals are few in number, and those available are lacking in detailed presentation of the results Moreover, they do not include quantitative microscopical methods, nor immunofluorescent investigations concerning immunoglobulin content in the glomeruli

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TABLE 4 *Ultrastructurally Measured Thickness of the Glomerular Basement Membrane in 3 Groups of 15 Month Old Male Rats*

Rat no	Electron microscopically measured mean thickness (Å)	Light microscopically graded thickness (1+, 2+, 3+, 4+)	Mean diuresis ml/100g/24h during the period 3–12 month of age
I Untreated control rats			
1	2980	3	
2	2840	3	
3	2880	3	
4	2870	3	
Mean±SD	2890±60		
II Untreated diabetic rats			
5	3540	3	43
6	3480	3	50
7	3570	3	74
8	3590	2	37
9	3200	3	61
Mean±SD	3480±160		53±15
III Insulin treated diabetic rats			
10	3460	2	59
11	3210	3	36
12	2760	3	50
13	3280	3	45
14	3320	3	88
Mean±SD	3210±270		56±20

Measuring method according to *Weibel & Knight* (33)
SD = standard deviation.

glomerular basement membrane thickness ($p < 0.01$), relative mesangial area ($p < 0.001$), and occurrence of IgG in the glomeruli ($p < 0.01$) IgG was mainly localized to the mesangium

A comparison of the light microscopical results obtained in the insulin treated and the untreated diabetic group (Tables 1 and 2) showed a slightly smaller basement membrane thickness and relative mesangial area in the former group but the differences were not statistically significant. Mesangial IgG (Table 3) occurred however, in significantly ($p < 0.01$) lower frequency and quantity in the insulin treated group Fluorescence for β_{1C} was not demonstrated with certainty in any rat.

The results of the ultrastructural measurements of the glomerular basement membrane thickness are presented in Table 4 The untreated diabetic rats had a significantly increased mean thickness compared with the nondiabetic control animals ($p < 0.001$) The mean of the insulin treated diabetic group was lower than that of the untreated group, but the difference was not significant ($0.05 < p < 0.10$)

If untreated control and insulin treated nondiabetic rats were compared, no significant differences between the groups could be demonstrated, neither regarding the glomerular basement membrane thickness, nor the mesangial area or the occurrence of glomerular IgG No β_{1C} was detected in the glomeruli

Intensely PAS-positive focal glomerular accumulations, resembling fibrinoid caps, were seen both in insulin treated and untreated diabetic rats These deposits occurred in about 40 and 30 per cent in the two groups

of rats respectively The frequency and severity of tubulo interstitial changes were of about the same magnitude in the two diabetic groups but they tended to be somewhat more severe in the insulin treated than in the untreated control group The type of tubular and interstitial changes observed was the same as that reported previously (17)

DISCUSSION

Since definite glomerular changes similar to diffuse glomerulosclerosis in human diabetics appear in rats with an untreated insulin deficiency diabetes (21), as described previously (17, 18 19), it was considered to be of interest to see whether insulin treatment might modify these changes As briefly mentioned in the Introduction insulin has been incriminated as a possible antigen involved in supposed immunological reactions that might be responsible for the development of microvascular disease in diabetes (1 2 5 10 14 23, 32 35) This hypothesis has arisen mainly from two observations ei

1 The binding of fluorescent insulin and anti insulin sera to small vessel walls in human diabetics (2, 3 10 13 35) Some of these individuals had not received insulin therapy (2 13) Formalin fixed paraffin embedded tissues were used in most cases

2 The induction of glomerular changes similar to human diabetic nephropathy in nondiabetic animals (rabbits and guinea pigs) by heterologous insulin injections (1, 6 21 23)

Some objections can be raised against these observations ei *ad 1* Some investigators using frozen unfixed kidney tissue were not able to confirm any unquestionable binding of insulin or anti insulin sera to renal vascular walls in diabetics (7 15 36) not even in insulin treated individuals (7 36) On the other hand two of the studies mentioned above where paraffin embedding was employed reported binding to vessel walls also in nondiabetics (3 13) Thus the results of immunohistochemical investigations of conjugated insulin and anti insulin sera appear at least to some extent to depend on the histological technique employed (*cf* 20) *ad 2* Heterologous insulin alone injected in animals has either elicited lesions lacking some of the characteristic features of diabetic glomerulosclerosis (12 16 31, 32) or failed to evoke any glomerular changes at all (23 25) Thickening of the glomerular basement membrane and clear-cut nodule like formations seem to have been induced in nondiabetic animals only when insulin has been given together with Freund's adjuvant (1, 6 21, 23)

The main argument against the hypothesis that exogenous insulin gives rise to glomerular disease and diabetic microangiopathy as a whole, is, however that microvascular lesions occur both in diabetic humans and animals who have never received insulin therapy (17, 18 30) It has been put forward that untreated diabetics might produce an antigenically altered endogenous insulin which would give rise to insulin antibodies (3) However, the existence of autoantibodies to endogenous insulin does not yet appear to have been convincingly demonstrated (*cf* 4) Another hypothesis proposes that diabetics produce complexes between insulin (exogenous or endogenous) and IgG not containing insulin antibodies. *In vitro* experiments have shown that when insulin and such IgG were reacted under mild reducing conditions a precipitate was formed which was highly reactive with complement (8) These complexes might produce or aggravate vascular lesions

As regards rats with experimental diabetes, only a few studies have been published where kidney structure was investigated both in untreated and insulin treated animals (9 26 27) A detailed description and comparison of the results obtained in the two groups were not presented however No definite conclusions concerning the effect of insulin on the glomeruli of the diabetic kidney can therefore be drawn from these studies *Spiro & Spiro* (29) found increased activity of glucosyl transferase an enzyme considered to be an indicator of basement membrane synthesis in

the kidney cortex of alloxan diabetic rats as compared with age-matched controls The difference between the normal and diabetic animals increased with the duration of the diabetes In insulin treated diabetic rats the enzyme level was normal or close to normal *Deb & Chakravarty* (11) reported the finding of several histochemical changes in the kidneys of rats with short term alloxan diabetes Most of the staining reactions were normal in diabetic animals given insulin

In the present study, alloxan diabetic male rats were given insulin therapy for 3 months after 9 months of untreated diabetes The insulin doses were adjusted so that their diabetic state, as controlled mainly by the amounts of urinary output and glucosuria, was markedly improved during the period of therapy Insulin treated diabetic rats presented a significantly lower frequency and smaller amount of mesangial IgG and a somewhat, but not significant, lower glomerular basement membrane thickness and mesangial area as compared with untreated diabetic animals of corresponding age and diabetes duration On the other hand, PAS positive hyaline glomerular deposits resembling fibrinoid caps, were at least as common in the insulin treated as in the untreated diabetic group Insulin injections in nondiabetic rats did not induce any obvious glomerular changes compared with untreated controls These results do not support the hypothesis that the presence of insulin is contributing to the development of the glomerulosclerosis in diabetes The findings are consistent with the concept that insulin deficiency, in some way or another causes glomerular lesions, including deposition of immunoglobulins

The present study has not clarified what mechanism causes IgG deposition in the glomeruli of diabetic rats However the demonstrated IgG can hardly represent antibodies to insulin The deposition of IgG is evidently a reversible phenomenon since almost all untreated alloxan diabetic rats exhibit glomerular IgG as early as one month after diabetes induction as demonstrated previously (19 Th disappearance of the immunoglobulins with a concomitant tendency to less glomerular basement membrane thickening and less mesangial widening in the insulin treated diabetic animals does not give any answer to the question whether the glomerular IgG plays any role for the development of the glomerulosclerosis in diabetes

This work was supported by grants from the *Medical Faculty, University of Umeå* the *Swedish Diabetes Association* and the *Nordic Insulin Fund*

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Acta path microbiol scand Section A 82, 235–248, 1974

THE FINE STRUCTURE OF A POSSIBLE CARCINOMA-IN-SITU IN THE SEMINIFEROUS TUBULES IN THE TESTIS OF FOUR INFERTILE MEN

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An abnormal morphology of germ cells was found in the seminiferous epithelium of four infertile men, three of whom developed a carcinoma of the same testis The ultrastructure of the abnormal germ cells from the different patients were compared and found to be almost identical The fine structure of the cells was also compared with that of the normal pre and postpubertal germ cells described in the literature We found that the abnormal germ cells were similar to the former although they also possesed several malignant features as regards the size and shape of nucleus nucleolus and the density of cytoplasmic organelles The ultrastructure of the Sertoli cells was compared with that of normal pre- and post pubertal Sertoli cells described in the literature Morphologically they seemed to be normal postpubertal Sertoli cells The findings indicate that the abnormal cells represent a carcinoma in situ of rather primitive germ cell origin

An abnormal morphology of the germ cells in testicular biopsies of two infertile men was described recently (*Skakkebæk* 1972 a) In addition to apparently normal Sertoli cells and a few apparently normal spermatogonia, one cell type only was found by light microscopy of the seminiferous epithelium

Both patients developed carcinoma of the testis within the observation period of 4½ years, and it was suggested that the originally described abnormal cells of the first testicular biopsies represented a carcinoma in situ (*Skakkebæk* 1972 b)

Recently two other infertile men with similar abnormal morphology of the cells of the seminiferous epithelium were found at the Fertility Clinic, and one of these had a testicular carcinoma

The aim of the present investigation was

1) to ascertain whether the abnormal cells, which were found to be identical by conventional histological examination, showed the same fine structure in the four patients,
2) to classify the abnormal cells by comparison with normal pre pubertal and postpubertal germ cells and Sertoli cells,
3) to investigate whether the abnormal cells had any characteristics of malignant cells,
4) to examine whether the histologically normal Sertoli cells in the otherwise abnormal tubules had a normal fine structure

Received 11 x 73 ... epted 11 x 73

GC
C
PC
CD
SC
1a
C
GC
C
1b
G
EG
NG
M
NS
ES
S
L
2

TABLE 1 *Histological Diagnosis of Cases in the Material*

e	Testis	1st biopsy	2nd biopsy	3rd biopsy	Orchiectomy
	Right	Nov 1967 Abnormal intratubular germ cells	* April 1972 Abnormal intratubular germ cells		* May 1972 Abnormal intratubular germ cells and early tumour development with invasion into interstitial tissue
	Left			* May 1972 Abnormal intratubular germ cells	* Aug 1972 Abnormal intratubular germ cells
	Right	*Oct 1970 Abnormal* intratubular germ cells and a few tubules with normal germ cells	* *Feb 1972 Abnormal* intratubular germ cells in a small area and embryonal carcinoma in the remainder		* *Feb 1972 Embryonal* carcinoma
	Left			July 1972 Sertoli cell only tubules	
	Right	June 1972 Abnormal intratubular germ cells	* Oct 1972 Abnormal intratubular germ cells		
	Left		* Oct 1972 Abnormal intratubular germ cells		
[illegible]	Right	Jan 1973 Seminoma			Jan 1973 Seminoma and a few Sertoli *cell-only tubules*
	Left	* Jan 1973 Abnormal intratubular germ cells and many hyalinized tubules			

* Biopsies included in the present study

Fig 1 a From case 1 A tubulus with large abnormal germ cells (GC) lying close to the basement membrane A few Sertoli cells are marked (SC) and the centre of the tubulus is filled with cell debris (CD) Collagen (C) and peritubular cells (PC) can be seen in the tubular wall There are a few Leydig cells in the lower right corner Vestopal imbedded 1 um thick toluidin blue stained section Magnification 350 ×

Fig 1 b From case 1 Part of an intact tubulus can be seen to the right La ge abnormal germ cells (GC) are found in the interstitial tissue There are also a few Leydig cells (LC Note the large amount of collagen (C n the interstitial t ssue Magnification 450

Fig 2 From case 3 Large abnormal germ cell without contact with the basement membrane and part of a Sertoli cell The ovoid nucleus (G) of the abnormal germ cell contains an evenly dispersed fine granular nucleoplasm and two fibrillar nucleoli (NG), but there are no chromatin granules on the inside of the nuclear envelope (EG) The cytoplasmatic organelles are few in number The mitochondria (M) have a swollen appearance The Sertoli cell *nucleus (S) with nucleolus (NS)* and a closely packed fine granular nucleoplasm have a thin layer of electron dense material on the inside of the nuclear envelope (ES) There are a few lipid granules (L) in the germ cell and many in the Sertoli cell cytoplasm Magnification 5 200 ×

MATERIAL AND METHODS

The four patients, who were of the ages 28 to 35 years, were discovered because of their infertility and were originally subjected to testicular biopsy because of severe oligospermia. Only case 4, whose right testis had a firm consistency and irregular shape, was suspected of having testicular carcinoma.

Three of the patients had normal descensus of the testis. Case 2 had descensus of the testis at the age of 9 years after repeated gonadotropin injections. All had normal puberty, normal urinary output of total gonadotropins and 17-ketosteroids, and normal karyotype, 46, XY. The size of the testicles varied from 12 to 27 cm^3 as measured by orchidometer.

As can be seen from Table 1, testicular biopsies were taken repeatedly from three of the patients. Bilateral orchiectomy was performed in one case and *unilateral orchiectomy was carried out in two* cases. The testicular biopsies were obtained surgically under local or general anaesthesia.

For electron microscopy, small blocks of tissue were fixed for about 3 hours in either Karnovsky's fixative (*Karnovsky* 1965) diluted 1:1 with distilled water, or in a cacodylate buffered mixture of glutaraldehyde, acrolein and formaldehyde (*Kalt & Tandler* 1971). After washing in cacodylate buffer and postfixation in cacodylate buffered 1 per cent osmium tetroxide for about 3 hours, they were dehydrated in graded concentrations of ethanol, followed by 100 per cent epoxypropane, and finally embedded in Vestopal W.

In order to get an outline of the tissue for electron microscopy, *1 μm sections* were made prior to and subsequent to the ultrathin sections. These *were stained with toluidine blue.*

Ultrathin sections were cut on a LKB Ultrotome and stained with magnesium uranyl acetate (*Frasca & Parks* 1965) and lead citrate (*Reynolds* 1963).

Electron microscopy was performed with a Siemens Elmiskope 1 A.

RESULTS

Light Microscopy of 1 μ Sections

The abnormal germ cells found by conventional histological examination in the seminiferous tubules of four infertile men (*Skakkebæk* 1972 a) were also identified by light microscopy of the tissue prepared for electron microscopy. In Cases 1, 3 and 4 intact tubules with abnormal cells were observed in non-carcinomatous tissue (Fig. 1 a). In Case 2, similar tubules were found only in testicular tissue which was changed due to embryonal carcinoma, and this tissue was not processed for electron microscopy. Islands of cells, similar to the abnormal germ cells, were found outside the seminiferous tubules in Case 1 (GC) (Fig. 1 b).

The seminiferous tubules were dominated by the large ovoid or round germ cells (GC) (Fig. 1 a). The cytoplasm of the largest germ cells was weakly stained *and often* had a "blown up" appearance, whereas the cytoplasm of the smaller cells usually was darker.

In addition to the unusually large germ cells, a few apparently normal A-spermatogonia were observed at the periphery of some tubules, and an occasional tubule with apparently normal spermatogenesis was seen in Case 1.

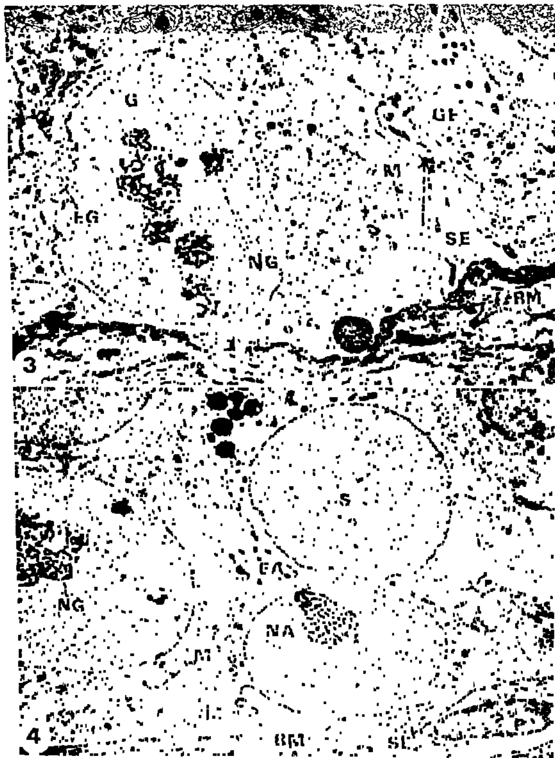
Apparently normal Sertoli cells were present in all tubules (SC) (Fig. 1 a). In comparison with the germinal cells, these were located more centrally in the tubules.

The central part of the *seminiferous tubules* was occupied by necrotic cells and cell debris (CD) (Fig. 1 a).

A few lymphocytes and macrophages were

Fig. 3. From case 1. Large abnormal germ cell with excentrically located nucleus (G) which displays an evenly dispersed fine granular nucleoplasm, several nucleoli (NG) and *an irregular nuclear* enveloped (EG). The cytoplasm is studded with organelles among which only mitochondria (M) are distinct. The cell has a broad contact area with the irregularly folded basement membrane (BM). Sertoli cell cytoplasm (SF) with tonofilaments (arrow) *and a few slender mitochondria* are seen. The cytoplasm of another abnormal germ cell is marked (GF). Magnification 5 200 ×.

Fig. 4. From case 3. The normal A-spermatogonium has a broad base against the basement membrane (BM). The area of contact is diminished by Sertoli cell cytoplasm (SF). The nucleolus (NA) is in contact with the nuclear envelope (EA). There are a few paired mitochondria in the cytoplasm (M). An abnormal germ cell with a large nucleolus (NG) and partly vesicle-shaped mitochondria (M) *is in close contact with the normal A-spermatogonium.* The nucleus of a Sertoli cell is marked (S). A nucleus of a peritubular cell (P) can be seen beneath the basement membrane. Magnification 5 000 ×.


G
GF
M
EG
SE
NG
BM
3
S
NG
NA
M
4
BM
SL
P

CR
ER
G
VG
GL
GO
RI
M
BM
5
L
RI
G
M
GM
6

seen frequently inside the seminiferous tubules.

The thickness of the tubular wall varied from one tubule to the next. It was often increased due to proliferation of the peritubular cells (PC) (Fig. 1 a) and/or to abnormal deposition of collagen (C) (Fig. 1 a). In the testis of Case 4, these changes were so pronounced that more than 90 per cent of the seminiferous tubules appeared in the form of cords of dense connective tissue.

Inconstant histological changes were observed in the interstitial tissue. Apparent Leydig cell hyperplasia was found only in biopsies from Cases 1 and 3 and pronounced lymphocyte infiltration in Case 1. In many biopsies, the interstitial tissue was the seat of abnormally large deposits of free collagen (C) (Fig. 1 b) as well as capillary wall thickening.

Electron Microscopy of Ultrathin Sections

1. *Germ Cells*

As regards fine structure, the germ cells had several morphological features in common and they always differed from the Sertoli cells. In order to facilitate the description, the germ cells were divided into two groups: A. abnormal germ cells, and B. apparently normal spermatogonia.

1A. Abnormal germ cells. The abnormal germ cells showed considerable variation with regard to cell size and shape, the diameter varying from 13 μ to 23 μ. Some of the cells which were located close to the wall of the seminiferous tubules had a flat shape while others were almost spherical. Among the latter were several which apparently had no contact with the basement membrane (Fig. 2). The area of direct contact between the flat germ cells and the basement membrane was sometimes small, since a thin layer of Sertoli cell cytoplasm was intercalated between the two.

Among the morphological findings, which were common to abnormal cells, were the excentrically placed nuclei with cytoplasmic organelles at the opposite pole (Fig. 3), the fine structure of nucleoplasm, nucleolus and nuclear envelope. The mitochondria from different cells had the same fine structure also and most cells contained annulate lamellae (AL) (Fig. 8).

The differences between the abnormal cells were most conspicuous in the actual or relative size of the nucleoli and in the relative number of the various cytoplasmic organelles. At the one extreme were cells with normal sized nucleoli and a cytoplasm which was sparsely equipped with organelles (Fig. 2). At the other extreme were cells with two or three enormous nucleoli and a cytoplasm studded with ribosomes, granulated endoplasmic reticulum and vesicles (Fig. 3 and 5).

The nuclei of the abnormal germ cells were round or slightly ovoid and had a smooth contour in some cells (EG) (Fig. 2) and a wrinkled contour in others (EG) (Fig. 3). They were approximately 12 μ in diameter. No heterochromatin was present, but sometimes a single layer of granules could be seen on the inside of the nuclear envelope (CR) (Fig. 5 and 8). The nucleoli were centrally placed in the nucleus. They sometimes occupied an area corresponding to one quarter to one third of the total area of the cross sectioned nucleus. The spongy nucleolonema displayed coarse granular areas as well as more amorphous areas.

The mitochondria were mostly ovoid (M) (Fig. 5 and 6) but rod shaped forms, often

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Fig. 5. *From case 3.* Part of an abnormal germ cell. The nucleus is marked (G). A small number of chromatin granules (CR) are visible inside the nuclear envelope. Among the densely packed cytoplasmic organelles there are granular endoplasmic reticulum (ER), free ribosomes (RI), glycogen granules (GL), Golgi vesicles (GO) and mitochondria (M). A few vesicles filled with electron dense material are marked (VG). Only a few lamellae of the basement membrane (BM) are visible. Magnification 27 000 ×.

Fig. 6. From case 3. Part of an abnormal germ cell with nucleus (G). Mitochondria (M) with a few tubular cristae (arrows) and one single lipid inclusion (L) can be seen. Free ribosomes are marked (RI) and an accumulation of fine electron dense granules is marked (GM). Magnification 38 000 ×.

of considerable length were observed. The smallest diameter was on the average 0.4 μ. The mitochondria were not located parallel to each other. The matrix was usually less dense than that of mitochondria of A spermatogonia (M) (Fig 2, 4, 5, 6), but in a few cells (especially from Case 1) it had increased electron density (M) (Fig 3) and almost completely concealed the cristae.

The large germ cells contained only few cisterns of the granular endoplasmic reticulum but as compared with the small A spermatogonia there was often a high density of free ribosomes mostly polysomes (RI) (Fig 5 and 6). Annulate lamellae were seen frequently. Three or four were located together sometimes communicating with the granular endoplasmic reticulum (AL) (Fig 8). The cytoplasm which surrounded the annulate lamellae and occupied the pores of it had increased electron density.

A few lipid like spherical cytoplasmic inclusions were present. These were not limited by a membrane and consisted of homogeneous material of moderate electron density (L) (Fig 2 and 6). Sometimes a few glycogen like granula were seen (GL) (Fig 5).

The Golgi zone was large and usually surrounded by a large number of cytoplasmic vesicles (GO) (Fig 5), some with electron translucent and some with electron dense contents. In the nearby cytoplasm but not directly associated with the membranous organelles small accumulations of granular electron dense material were seen occasionally (GM) (Fig 6).

A few cytoplasmic microtubules (MT) (Fig 8) were present in the large germ cells and lysosome like structures consisting of membrane conglomerates were common.

1B Normal spermatogonia. Apparently normal A spermatogonia were flat cells measuring about 8 μ by 18 μ located close to the wall of the seminiferous tubules (Fig 4), sometimes separated from the basement membrane only by a 160 Å space, sometimes by a thin strand of Sertoli cell cytoplasm (SE) (Fig 4).

The nucleus was round or ovoid with a diameter of about 8.5 μ. It showed an evenly dispersed fine granular nucleoplasm and usually one nucleolus (NA) (Fig 4) which was located close to the nuclear envelope (EA) (Fig 4). The nucleolonema and pars amorpha were usually distinct. No accumulation of chromatin was seen on the inner surface of the nuclear envelope.

The cytoplasm contained only few organelles. Dominating were mitochondria with a round or ovoid shape (M) (Fig 4 and 7) averaging 0.4 μ on the short diameter. They were sometimes arranged in pairs with the long axis parallel to each other (M) (Fig 7). The space between them was 0.1 μ wide, often with a thin layer of electron dense material (Arrow Fig 7). The cristae mitochondrias were few and irregular in shape and many tubular forms could be seen. The mitochondrial matrix was of moderate density. The A spermatogonia contained one Golgi region which was surrounded by a few vesicles and a few profiles of granular endoplasmic reticulum. Short strands of the latter were often found close to the mitochondria. There were only few free ribosomes in the cytoplasm. Crystals of Lubarsch (LC) (Fig 7) and annulate lamellae (AL) (Fig 7) were observed occasionally.

A few cells found in Case 3 had the same fine structure as the A spermatogonia but both the cell body and the nucleus had a more spherical shape. Only a small part of the surface was in contact with basal lamina.

No normal B spermatogonia, spermatocytes

Fig 7. From case 1. Detail from a normal A spermatogonium. The nucleus is marked (A). Two mitochondria (M) are lying side by side separated only by a layer of electron dense material (arrow). Crystal of Lubarsch (LC) and perpendicularly cut annulate lamellae (AL) are visible. (MT) denotes microtubules. Magnification 34 000.

Fig 8. From case 3. Detail of an abnormal germ cell. The nucleus (G) has chromatin granules (CR) displaced towards the nuclear envelope (EG). *Oblique and tangentially sectioned cisterns* of annulate lamellae (AL) are connected with the granular endoplasmic reticulum (ER). Many microtubules (MT) are present. Magnification 39 000 ×.

M
A
MT
LC
AL
7
G
CR
EG
AL
AL
MT
ER
8


G
LY
NG
9
G
G
L
M
BM
M
10

or spermatids were found in the abnormal tubules

2 *Sertoli Cells*

The Sertoli cells encircled the germ cells and were in contact with the basement membrane The ovoid nucleus was approximately 8 5 μ in diameter It often showed a deep indentation of the nuclear envelope The nucleoplasm had a fine granular texture and contained no visible chromatin A condensation of electron dense granules (ES) (Fig 2) was always present on the inside of the nuclear envelope The nucleolus had a central location and consisted usually of a prominent round pars amorpha which was surrounded by a coarse granular and a fine fibrillar nucleolonema

Some parts of the Sertoli cell cytoplasm were dominated by a dense network of tonofilaments (arrow Fig 3), others by large accumulations of lipid like inclusions (L) (Fig 2) Long slender mitochondria with a dense matrix and regular shaped christae were distributed evenly in the cytoplasm Their small diameter was about 0 3 μ The cytoplasm also contained a smooth endoplasmic reticulum and a well developed Golgi zone Organelles resembling residual bodies containing a varying amount of cell debris were frequently observed in the cytoplasm which extended towards the centre of the seminiferous tubules

Fig 9 Interstitial tissue from Case 1 Part of an abnormal germ cell with a large excentrically placed nucleus (G) and two nucleoli (NG) The nucleoplasm of a Leydig cell (LN) has a coarse granular texture and chromatin (CR) is deposited on the inner aspect of the nuclear membrane The Leydig cell cytoplasm is densely packed with mitochondriae lipid inclusions (L and electron translucent vesicles Two lymphocytes are marked (LY) Magnification 46 000

Fig 10 Interstitial tissue from Case 1 Part of two abnormal germ cells with nuclei marked (G) A single lipid like inclusion (L) and many free ribosomes (RI) are visible (M) denotes mitochondriae A single lamella of basement membrane (BM) is deposited on the surface of the two abnormal germ cells Magnification 23 000 ×

3 *The Wall of the Seminiferous Tubule*

The wall of the seminiferous tubule includes the basement membrane and alternating layers of collagen fibres and tubular wall cells

Occasionally lymphocytes and/or plasma cells were present in the wall close to the basement membrane and also inside the tubule. The basement membrane consisted normally of 6–8 electron dense lamellae The lamellae were approximately 250 Å wide (BM) (Fig 5) but had no sharp boundary Occasionally small vesicles were observed in the empty space between the lamellae

The basement membrane was sometimes folded (BM) (Fig 3) and the projections formed extended centrally between the germ cells and/or the Sertoli cells Some parts of the basement membrane had no substructure of lamellae but were formed of homogeneous moderately electron dense material

Large masses of abnormally twisted and curling collagen fibres were seen frequently in the tubular wall Usually no tubular wall cells were present within these masses of collagen Peritubular cells (P) (Fig 4) were situated between bundles of collagen fibres and ramifications of their cytoplasm appeared in the form of thin strands The flat nucleus with abundant chromatin material was often crumpled The perinuclear cytoplasm usually contained a well developed Golgi apparatus several layers of endoplasmic reticulum, many free ribosomes, many pinocytose vesicles and slender mitochondria Several microtubules, but only few microfilaments were present in the cytoplasm A few lipid like inclusions were seen

4 *Abnormal Germ Cells in the Interstitial Tissue*

Clusters of abnormal germ cells were found outside the seminiferous tubules in the right testis of Case 1 (Fig 9) The fine structure was identical with that of the intratubular abnormal germ cells, except that the nucleus was more often placed centrally in the cell and that they had a higher nucleo/cytoplasmic ratio The extratubular germ cells



G
LY
LN
CR
NG
LY
9
G
G
L
M
RI
BM
M
10

or spermatids were found in the abnormal tubules

2 *Sertoli Cells*

The Sertoli cells encircled the germ cells and were in contact with the basement membrane The ovoid nucleus was approximately 8 5 μ in diameter It often showed a deep indentation of the nuclear envelope The nucleoplasm had a fine granular texture and contained no visible chromatin A condensation of electron dense granules (ES) (Fig 2) was always present on the inside of the nuclear envelope The nucleolus had a central location and consisted usually of a prominent round pars amorpha which was surrounded by a coarse granular and a fine fibrillar nucleolonema

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3 *The Wall of the Seminiferous Tubule*

The wall of the seminiferous tubule includes the basement membrane and alternating layers of collagen fibres and tubular wall cells

Occasionally lymphocytes and/or plasma cells were present in the wall close to the basement membrane and also inside the tubule The basement membrane consisted normally of 6–8 electron dense lamellae The lamellae were approximately 250 Å wide (BM) (Fig 5) but had no sharp boundary Occasionally small vesicles were observed in the empty space between the lamellae

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4 *Abnormal Germ Cells in the Interstitial Tissue*

Clusters of abnormal germ cells were found outside the seminiferous tubules in the right testis of Case 1 (Fig 9) The fine structure was identical with that of the intratubular abnormal germ cells except that the nucleus was more often placed centrally in the cell and that they had a higher nucleo/cytoplasmic ratio The extratubular germ cells

were partly separated from the surrounding connective tissue by a single layer of basement membrane (BM) (Fig 10)

DISCUSSION

The morphology of the abnormal germ cells from the different patients was almost similar from the point of view of cell size the ex centrical location of the nucleus and the fine structure of mitochondria nuclear envelope and nucleoli The cells also possessed a well developed system of annulate lamellae accu mulations of fine electron dense granules and a few lipid like inclusions in the cytoplasm They share most of these morphological char acteristics with normal gonocytes and pre spermatogonia (*Gondos & Hobel* 1971 *Gondos & Conner* 1973 *De Kretser* 1968, *Wartenberg et al* 1971) described in the litera ture and hence the findings in the present study strongly support the assumption that the abnormal cells are of germ cell origin (*Skakkebæk* 1972 a)

It was evident that the abnormal germ cells were not developed from Sertoli cells as they had none of their gross morphological characteristics and were also distinctly differ ent as regards the fine structure of mito-chondriae nuclear envelope nucleoplasm and nucleoli (*Bawa* 1963 *Fawcett & Burgos* 1956a *Nagano* 1966 *Soh et al* 1971) Both cell types contained lipid inclusions in the cyto-plasm but they were found to be far more numerous in the Sertoli cells In addition only the Sertoli cells contained large numbers of microfilaments in the cytoplasm

The abnormal germ cells differed from normal A and B spermatogonia (*Burgos et al* 1970 *Nagano* 1969 *Rowley et al* 1971 *Soh et al* 1971 *Tres & Solari* 1968) in that they were larger and had a larger nucleus and nu cleolus Sometimes they also had a higher density of cytoplasmic organelles especially mitochondriae free ribosomes and granular endoplasmic reticulum

The fine structure differed particularly from the fine structure of B spermatogonia in that the nucleus of normal B spermatogonia is located centrally in the cell and contains coarse granular chromatin flakes whereas the abnormal germ cells mostly had an excentri cally placed nucleus and a homogeneous fine granular nucleoplasm As far as we know, the annulate lamellae (*Kessel* 1968) often ob served in the abnormal germ cells have not been seen in human B spermatogonia (*Burgos et al* 1970 and *Rowley et al* 1971)

As regards cell shape and mode of contact with the basal membrane many abnormal cells were more like A spermatogonia as they often had a flat cell shape and were located close to the basal membrane We are there fore inclined to believe that the abnormal germ cells originate from germ cells at an earlier developmental stage than B sperma togonia, i e A spermatogonia or more prob ably gonocytes or prespermatogonia A find ing supporting the hypothesis that they were maldeveloped gonocytes or prespermatogonia is that the abnormal cells in several respects were similar to developing gonocytes of hu man embryos (*Gondos & Hobel* 1971, *Wartenberg et al* 1971) This applies both for their size and shape and to the fine structure of the cytoplasmic organelles The nucleus was even larger than that of normal gono cytes (unpublished observation)

The higher density of cytoplasmic organ elles in the abnormal germ cells as compared with normal spermatogonia indicates that the former have a higher metabolism and growth rate than normal germ cells The enlarged nucleolus also points in that direction The larger nuclear and cytoplasmic volume of the abnormal cells are compatible with the previ ous finding (*Skakkebæk* 1972 b) that many of them are almost tetraploid which may indicate irregular cell division The cells thus have sev eral morphological features of anaplasia and may therefore be malignant or premalignant cells The fact that the mitochondriae in the abnormal germ cells often had a more dense n

swollen

adjacent

assumptio

cation of malignancy is the finding of ab normal germ cells outside the miniferous

tubules Some of the extratubular germ cells were similar to the abnormal germ cells inside the tubules, although others had a centrally located nucleus and a higher nucleo/cyto plasmic ratio

It is not known whether these interstitial germ cells were derived from intratubular germ cells or whether they were developed from primitive germ cells which were left outside the seminiferous tubules during the development of the testis However, the fact that a testicular biopsy performed 4½ years earlier in Case 1 did not show any germ cells in the interstitial tissue indicates that the interstitial germ cells originated from intra tubular cells

Usually the extratubular germ cells were easily distinguishable from Leydig cells because of the different composition of the cytoplasm the presence of a much larger nucleus, and the absence of accumulations of peripheral chromatin material which is characteristic of Leydig cells (*Fawcett* & *Burgos* 1956 b, *Fawcett* & *Burgos* 1960, *De Kretser* 1967 a) The annulate lamellae which were also found in the extratubular germ cells have, to our knowledge, never been observed in Leydig cells

It seemed that only the germinal cells were changed in the abnormal seminiferous tubules The Sertoli cells showed no deviation from the normal ultrastructure, nor had they the appearance of immature Sertoli cells (*De Kretser* 1968) Thus, any morphological support for the assumption that development of the abnormal germ cells was the result of abnormal Sertoli cell function was not found, as might have been expected from the presumed great significance of Sertoli cells in normal germ cell functions (*Ross* 1971, *Vilar et al* 1962)

We conclude that this investigation has demonstrated the mutual morphological identity of the abnormal germ cells from the 4 patients They had many characteristics in common with germ cells in general, especially with prepubertal germ cells and they were distinctly different from the Sertoli cells all of which had a normal fine structure The abnormal germ cells had several morphological features of malignancy and thus, they may represent a carcinoma-in situ of rather primitive germ cells

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were partly separated from the surrounding connective tissue by a single layer of basement membrane (BM) (Fig 10)

DISCUSSION

The morphology of the abnormal germ cells from the different patients was almost similar from the point of view of cell size, the ex centrical location of the nucleus, and the fine structure of mitochondria, nuclear envelope and nucleoli The cells also possessed a well-developed system of annulate lamellae, accu mulations of fine electron dense granules, and a few lipid like inclusions in the cytoplasm They share most of these morphological characteristics with normal gonocytes and pre spermatogonia (*Gondos & Hobel* 1971, *Gondos & Conner* 1973 *De Kretser* 1968, *Wartenberg et al* 1971) described in the literature and hence, the findings in the present study strongly support the assumption that the abnormal cells are of germ cell origin (*Skakkebæk* 1972 a)

It was evident that the abnormal germ cells were not developed from Sertoli cells as they had none of their gross morphological characteristics and were also distinctly different as regards the fine structure of mito chondriae, nuclear envelope, nucleoplasm, and nucleoli (*Bawa* 1963, *Fawcett & Burgos* 1956a *Nagano* 1966, *Sohval* 1971) Both cell types contained lipid inclusions in the cytoplasm but they were found to be far more numerous in the Sertoli cells In addition, only the Sertoli cells contained large numbers of microfilaments in the cytoplasm

The abnormal germ cells differed from normal A and B spermatogonia (*Burgos et al* 1970 *Nagano* 1969, *Rowley et al* 1971 *Sohval* 1971, *Tres & Solari* 1968) in that they were larger and had a larger nucleus and nucleolus Sometimes they also had a higher density of cytoplasmic organelles especially mitochondriae free ribosomes and granular endoplasmic reticulum

The fine structure differed particularly from the fine structure of B spermatogonia in that the nucleus of normal B spermatogonia is located centrally in the cell and contains coarse granular chromatin flakes whereas the abnormal germ cells mostly had an excentrically placed nucleus and a homogeneous fine granular nucleoplasm As far as we know the annulate lamellae (*Kessel* 1968) often observed in the abnormal germ cells have not been seen in human B spermatogonia (*Burgos et al* 1970 and *Rowley et al* 1971)

As regards cell shape and mode of contact with the basal membrane, many abnormal cells were more like A spermatogonia as they often had a flat cell shape and were located close to the basal membrane We are therefore inclined to believe that the abnormal germ cells originate from germ cells at an earlier developmental stage than B spermatogonia, i e A-spermatogonia or more probably gonocytes or prespermatogonia A finding supporting the hypothesis that they were maldeveloped gonocytes or prespermatogonia is that the abnormal cells in several respects were similar to developing gonocytes of human embryos (*Gondos & Hobel* 1971, *Wartenberg et al* 1971) This applies both for their size and shape and to the fine structure of the cytoplasmic organelles The nucleus was even larger than that of normal gonocytes (unpublished observation)

The higher density of cytoplasmic organelles in the abnormal germ cells as compared with normal spermatogonia indicates that the former have a higher metabolism and growth rate than normal germ cells The enlarged nucleolus also points in that direction The larger nuclear and cytoplasmic volume of the abnormal cells are compatible with the previous finding (*Skakkebæk* 1972 b) that many of them are almost tetraploid which may indicate irregular cell division The cells thus have several morphological features of anaplasia and may therefore be malignant or premalignant cells The fact that the mitochondriae in the abnormal germ cells often had a more swollen appearance than mitochondriae in adjacent normal germ cells may support this assumption (*Bernhard* 1969) Another indication of malignancy is the finding of abnormal germ cells outside the seminiferous

Acta path microbiol scand Section A 82, 249 254, 1974

HISTOLOGICAL CHANGES IN MOUSE LYMPH NODES AND SKIN FOLLOWING REPEATED SKIN PAINTING WITH OXAZOLONE

Preliminary Report

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The response of lymph nodes and skin to repeated topical application of oxazolone was investigated histologically in mice The initial paracortical response in the nodes was followed by a prolonged germinal centre reaction and the development of numerous plasma cells The epidermis which initially showed an acute inflammatory reaction with erosion, healed despite repeated painting

The morphological response of draining lymph nodes to painting of the skin with 2-phenyl-4 ethoxymethylen 5-oxazolone (oxazolone) has been thoroughly investigated in guinea pigs by *Turk & Stone* (1963), *Oort & Turk* (1965), and in mice by *Fjelde & Turk (1965), Parrot* (1967), and *Davies et al* (1969) In the above mentioned works the reaction to a single application of oxazo lone was studied and the time of observation after oxazolone painting did not exceed 10 days

The purpose of the present investigation was to study the histological changes in the draining lymph nodes and skin of mice exposed to repeated application of oxazolone for a period of up to 5 weeks

MATERIAL AND METHODS

Animals

8 12 week old female mice of the closed colony kept at this Institute (*Hartveit* 1961) were used

Oxazolone in a 10 per cent alcoholic solution was painted on the skin at a dose of 0 1 ml per application Painting was performed twice weekly at three day intervals.

Two experimental groups were studied

In one containing 18 animals, the *abdominal skin* and *inguinal lymph nodes* were examined after painting the lower part of the abdomen Three animals were sacrificed on days 4 and 7 after the start of the experiment, thereafter weekly for 5 weeks each time four days after the last painting with oxazolone

In another group containing 10 animals the *auricular lymph nodes* were studied after painting of both ears at similar intervals These mice were sacrificed after 5 weeks

Control animals were 10 untreated mice of the same age and sex From these the inguinal and auricular lymph nodes, as well as the abdominal skin were examined histologically

The tissues were fixed in 4 per cent buffered formalin and paraffin embedded Sections for histology were stained with haematoxylin and eosin, methyl green pyronine and Giemsa stains

RESULTS

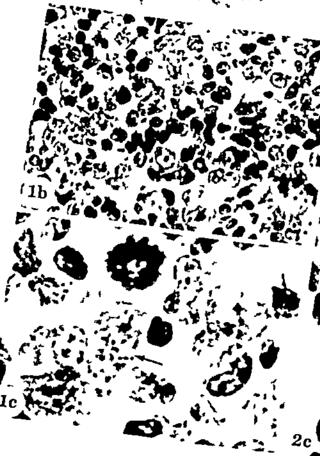
Inguinal Lymph Nodes

Four days after a single painting with oxazolone, marked enlargement of the lymph nodes

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1a


2a


1b


2b
2c


1c

was seen in all the mice compared to the untreated controls This was due to a diffuse paracortical hyperplasia The paracortical areas contained numerous pyroninophilic blast cells, which showed moderate basophilia of the cytoplasm with Giemsa stain Many mitoses were observed (Figs 1a 1b and 1c) The germinal centres were few and small

On day 7 a marked germinal centre reaction ensued, as well as marked hyperplasia of the medullary cords The germinal centres were large and often irregular in outline, characteristically confined to the cortical area, but often deeply situated Some hyperactivity of the paracortical areas was seen

On the 14th day the paracortical areas were either normal or atrophic, often surrounded or replaced by hyperplastic medullary cords and germinal centres In some *nodes the paracortical areas could scarcely be* found If present, the number of pyroninophilic blast cells did not usually exceed the numbers seen in inguinal lymph nodes from untreated controls In some, a slight hyperactivity was found The medullary cords contained tightly packed pyroninophilic plasma cells the cytoplasm being deeply basophilic with Giemsa stain This pattern persisted during the rest of the experiment and no further changes were seen (Figs 2a, 2b, and 2c)



Fig 3 Auricular lymph node after 5 weeks painting of both ears with oxazolone twice weekly Well developed germinal centres in the cortex The small paracortical area (arrows) is surrounded by medullary cords Methyl green pyronine × 15

Fig 1 Inguinal lymph node 4 days after a single painting of oxazolone on the abdominal skin

a) The paracortical area is prominent the cortex narrow There are a few small germinal centres in the cortex Giemsa × 15

b) Paracortical area showing abundant blastoid cells and cells in mitosis (arrows) Giemsa × 600

c) Paracortical area with well-developed blastoid cells Two cells in mitosis and one in a premitotic phase (arrow) Moderate basophilia of the cytoplasm of blastoid cells Giemsa × 1500

Fig 2 Inguinal lymph node after 5 weeks of oxazolone painting of the abdominal skin twice weekly

a) Well-developed germinal centres in the cortex and deeper among the medullary cords Hyperplasia of the medullary cords and atrophy of the paracortical area Methyl green pyronine × 15

b) Plasma cells in the medullary cords Methyl green pyronine × 600

c) Mature plasma cells in the medullary cords The cytoplasm is deeply basophilic with perinuclear halos Giemsa × 1500

Auricular Lymph Nodes

In the untreated controls these lymph nodes were scarely visible to the naked eye, but could be detected in serial sections of the loose connective tissue and fatty tissue extending from the base of the ear to the submaxillary region They usually contained a few follicles and a few blast cells in the paracortical areas In the 10 mice studied *after 5 weeks of skin painting* marked enlargement of the nodes was seen One or two nodes were found on either side They were easily dissected free from the surrounding tissue

The histological appearance of these enlarged nodes was similar to the pattern seen in the inguinal lymph nodes after the first week and during the rest of the experiment Large and irregular germinal centres were present as well as marked hyperplasia of the medullary cords which contained tightly packed plasma cells The paracortical area was in most instances atrophic (Fig 3)

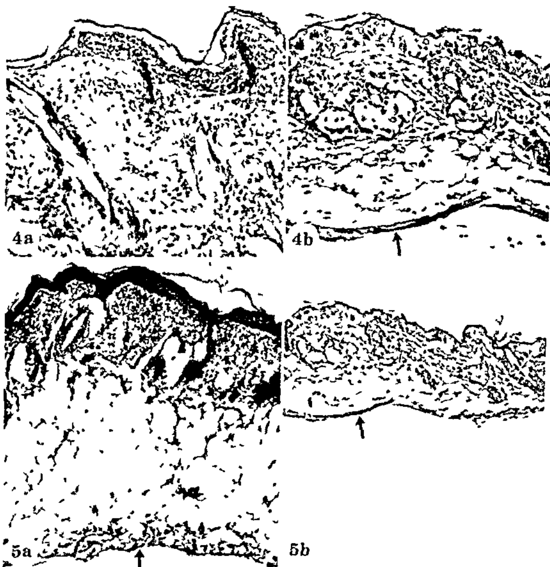


Fig 4 H+E × 100
a) Abdominal skin at 4 days after a single painting with oxazolone Intra epidermal accumulation of granulocytes with degeneration of the upper part of the epidermis Marked oedema in the dermis. Compare with fig 4b showing untreated abdominal skin The arrow points to the panniculus carnosus

Fig 5 H+E × 50
a) Abdominal skin after 3 weeks oxazolone painting twice weekly Complete regeneration of the epidermis hypercellularity of the dermis and marked oedema Compare the distance from the surface to the panniculus carnosus (arrow) with that of fig 5b showing untreated abdominal skin

Skin

The abdominal skin examined *4 days* after painting showed patchy necrosis of the epidermis with focal areas of regeneration in its deeper parts There was marked oedema in the dermis with neutrophil granulocytes in its upper parts and in the epidermis *A few* sub epidermal bullae were seen Erosions or ulcers were not found (Fig 4a) By *day 7* desquamation of necrotic epidermal tissue and regeneration of the epidermis was seen The regenerated epidermis appeared quite

normal with the exception of a few shallow erosions. After *two weeks* no erosions or ulcers were found. Marked oedema and infiltration of mono-nuclear cells was seen in the dermis. This picture remained unaltered during the rest of the experiment (Fig. 5a). Macroscopically, the fur disappeared at the application site at the end of the first week. After two weeks partial regeneration had occurred.

DISCUSSION

The morphological response of skin and draining lymph nodes to repeated topical application of oxazolone was followed in mice for 5 weeks. In the lymph nodes the initial paracortical reaction declined during the second half of the first week. Thereafter a marked germinal centre reaction and hyperplasia of the medullary cords containing tightly packed plasma cells dominated during the rest of the experiment. No recurrence of the paracortical reaction was seen subsequent to further application of oxazolone. The epidermis that initially showed an acute inflammatory response with desquamation and erosions regenerated during the second half of the first week of the experiment.

The initial paracortical reaction is in keeping with previous findings in guinea pigs and mice following a single topical application of oxazolone (*Turk & Stone* 1963, *Oort & Turk* 1965, *Parrot* 1967, *Davies et al.* 1969).

Reports on plasma cell development in the medullary cords have previously been contradictory. *Turk & Stone* (1963) and *Oort & Turk* (1965) described the appearance of plasma cells in lymph nodes of the guinea pig on the 6th day. *Davies et al.* (1969) in a study of mice found germinal centre formation and development of plasma cells synchronously with the decline in the numbers of blast cells in the paracortex from day 4 and onwards. At day 10 the paracortex was normal while hyperplasia persisted in the follicles, to a lesser extent in the medullary cords. However, plasma cell formation and the development of germinal centres was not observed by *Fjelde & Turk* (1965) after an equally long time of observation in mice. In the present study, in which skin painting was repeated twice weekly and the lymph nodes studied 4 days after the last application of oxazolone, well developed germinal centres and hyperplasia of the medullary cords were seen after 1 week and persisted during the rest of the experiment. The medullary cords contained tightly packed plasma cells. No recurrence of the initial paracortical reaction was observed. The findings in the auricular lymph nodes examined after 5 weeks were similar to the pattern seen in the inguinal lymph nodes after the first week and onwards.

The skin reaction initially showed an acute inflammatory response in the epidermis and in the upper part of the dermis. This has previously been described by *Davies et al.* (1969) and by *Dietrich & Hess* (1970). In spite of repeated painting with oxazolone the epidermis regenerated and appeared normal after 2 weeks. However, marked oedema and cellular infiltration persisted in the dermis.

Paracortical hyperplasia in lymph nodes has been shown to be thymus dependent (*Parrot* 1967, *Davies et al.* 1969), and associated with the delayed type of immune reaction. Germinal centre formation and plasma cell development is associated with production of antibodies (*Turk* 1969). The decline of the paracortical reaction observed in this study as well as the marked germinal centre reaction with numerous plasma cells indicate that the initial delayed type of immune reaction may turn into antibody production, the reaction being no longer thymus dependent. While skin infection also is a possible cause for the plasma cell reaction, this possibility seems less likely as the plasma cell reaction persisted far beyond the time taken for regeneration of the epidermis. The nature of the lymph node changes here described is under further investigation.

The skilled technical assistance of Mrs. *Anne marie Sundstrøm*, financed by the *Norwegian Cancer Society*, is gratefully acknowledged.

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Acta path. microbiol scand Section A 82, 255 263, 1974

LIPID DROPLETS IN FATTY LIVER

An Electron Microscopic Study on Ultrasections Obtained by a New Cutting Technique

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A modified method for cutting thin section for electron microscopic studies from tissue rich in lipid is presented Cutting procedure was performed at 8° (C) and results in flat sections and larger areas sectioned Liver biopsy specimens from 9 patients with fatty liver are studied A highly varied morphology of the lipid vacuoles is revealed Droplets with triglyceride, vacuoles emptied of fat, some with a fine network and a capsule like arrangement, and transition stages between the two patterns are studied electron microscopically and histochemically by OTAN and Oil Red O staining In some of the droplets small, dense granules of varied size and morphology are found

The ultrastructural alterations of the hepatocytes in fatty liver, especially the alcoholic fatty liver, have been extensively studied (*Rubin & Lieber* 1967), but informations of structure of the lipid droplets are scarce

Tissues rich in lipid are difficult to cut for electron microscopy, and fat accumulated in droplets or cysts as in fatty liver often present "chatter" or "wash boarding" These artefacts do not only prevent electron microscopic evaluation of the droplet or cyst, but also of the nearest hepatocytes Therefore a technique by which even the most fatty infiltrated areas could be cut without "chatter" was developed This technique and its application on human fatty livers in connection with histochemical investigations is presented in the present paper

MATERIAL

Liver biopsies were performed by the Menghini technique in 9 patients where steatosis were suspected from clinical and biochemical findings The diagnosis was confirmed by light microscopy at a part of the biopsy specimen fixed in neutral formalin In seven of the patients high alcohol consumption was supposed to be the cause of the steatosis and in two patients maturity onset diabetes was the most likely cause Three of the patients had slight steatosis and six had moderate steatosis as judged by light microscopy There were no signs of cirrhosis in any of the biopsies

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METHODS

The liver biopsy was divided in three pieces one for formalin fixation for light microscopy, another was fixed in cold formol calcium for 24 h, for histochemical preparations embedded in gum arabic, and cut in 10 μ sections in a cryostat The third part of the specimen was immediately divided into pieces of 0 5 mm length and fixed in cold cacodylate buffered 2 5 per cent glutaraldehyd for 2 h, followed by washing in 0 2 M sodium cacodylate, fixation in cacodylate buffered 1 per cent osmium tetroxide followed by dehydration in alcohol and propylenoxide before embedding in EPON

The 10 μ thick sections for histochemical reactions were stained with Oil Red O for demonstration of lipid and OTAN (osmium tetroxide alfa naphtyl) for demonstration of triglyceride and phospho-lipid (*Adams* 1965)

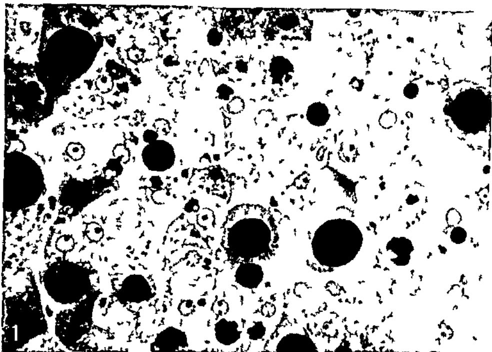
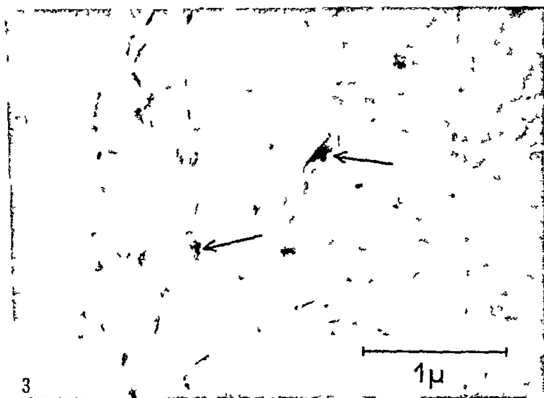


Fig 1 Droplets of fat in human liver biopsy 1 μ thick EPON embedded section stained with toluidine blue × 500



Fig 2 Hepatocytes with lipid droplets empty of fat A fine network is seen in the droplets 1 μ thick EPON embedded section stained with toluidine blue × 300



F g 3 Part of a fat droplet w h nhomo₀eneous dens ty In tl e per phery a concentr c capsule l ke arrangen ent s seen Note the small very electron dense granules (arrows) Electron m crograph Sta ned w th z nc uranyl acetate and lead c trate × 36 000

In order to keep the EPON embedded b opsy spec men cooled dur ng cutt ng b opsy spec men l ram cro ome (LKB) glass kn es and water n the boats on the glass kn ves v ere cooled by plac ng the ul ram crotome n a room w th a tem perature of 8° C ten hours before sect on ng was performed The th ckness of the ultrasect ons was 300–600 Å The sect ons were collected on gr ds mounted w th parlod on and carbon films and sta ned v tl z nc uranyl acetate and lead c trate (*H e nste n et al* 1963) Adjacent 1 μ sect ons were used for l ght m croscopy after sta ı ng w th tolu d ne blue

RESULTS

By the cold cutt ng tecl nique the following advantages were obta ned 1) flat tl ın sections could be cut even ıf the b opsy con tained much fat 2 Larger areas could be cut somet mes sq ares w th s des more than 0 5 mm of lengtl which opened a possibility of evaluating larger areas ın the electron m croscope correlated to the survey sections 3) Thereby an evaluation of many fat accu mulations was possible resulting surprisingly ın detection of a highly varied morphology of the lip d droplets If temperature rose to 14° C cutting was not satisfactory

In the survey sect ons most of the l pid droplets were strongly osmioph lic (Fig 1) while some were not (Fig 2) The droplets varied greatly ın size with a diameter from 0 5 to 30 μ In the not osmıophil c vacuoles fine networks sta nable with tolud ne blue were seen

Ultrastructurally the osm ophil c fat drop lets often appeared to contain moderately electron dense material of a homogenous nature Just as common electron dense ma

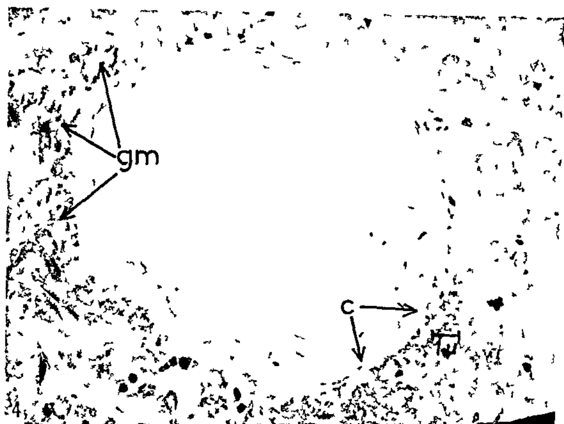


Fig 4 A hepatocyte containing a lipid vacuole with a fine network In the periphery this is arranged in a capsule like pattern (c) The mitochondria of the cell are enlarged some of them giant mitochondria (gm) Electron micrograph Stained with zinc uranyl acetate and lead citrate × 5 850

terial appeared in an irregular arrangement and in such droplets a concentrically arranged layer of this osmiophilic material was seen in the periphery with less electron dense areas in between In this capsule like arrangement a great deal of strongly electron dense granules 100 Å 1000 Å of size were observed (Fig 3) They were also to a lesser degree found near the center of the droplet

Corresponding to the not osmiophilic vacuoles seen in the survey sections not osmiophilic vacuoles were found electron microscopically too (Fig 4) In these vacuoles a fine network was found By high magnification it seemed not membrane like (Fig 5) and displayed near the periphery a capsule like arrangement in which electron dense granules reminiscent to those in the osmiophilic droplets were observed Many droplets showed transition stages between the two described patterns (Fig 6) and many droplets without osmiophilic material or structures were seen too

The 10 µ thick cryostat sections stained with Oil Red O showed lipid in the droplets but also by this staining method a heterogeneous staining could be seen in many of the droplets (Fig 7)

OTAN staining showed triglyceride in many of the lipid vacuoles but in some staining was lacking especially in the centre and in a few a roughly meshed network was found (Fig 8) Moreover Oil Red O and OTAN staining revealed fat and triglyceride between some hepatocytes (Fig 9 and 10)



Fig 5 Part of a lipid droplet presenting a finely granular network Note the small very electron dense granules (arrows) Electron micrograph Stained with zinc uranyl acetate and lead citrate × 45 000

DISCUSSION

Human fat consisting mainly of triglyceride melts at a temperature of approximately 17° C and is therefore fluid at normal room temperature

In liver biopsy specimens prepared for electron microscopic studies some of the triglyceride will be extracted before and during embedding in EPON The remaining triglyceride is fluid during ordinary sectioning conditions By cooling the biopsy specimen to 8 10° C an improved cutting of the fat droplets was achieved Furthermore it was possible to obtain larger tissue areas which gave a better survey

Electron microscopically the fat droplets displayed a slight to moderate homogeneous electron density but just as often an irregular arrangement of [illegible] with great electron density alternating with parts with a less pronounced electron density was observed This is presumably the same picture as described by *Oberling et al* (1956) in rat liver after carbon tetracloride poisoning This corresponded to the toluidine blue stained droplets in the survey sections The droplets not stained with toluidine blue corresponded to droplets not stained with OTAN These droplets must be considered to be depleted of triglyceride corresponding to the elctron lucid droplets These empty droplets presented in some cases a network visualized as well electron microscopically as histochemically by OTAN and toluidine blue staining

It may therefore by thought that some vacuoles have been depleted of triglyceride perhaps presenting one of the latest stages of the droplets

By OTAN and Oil Red O staining it has



Fig 6 Part of a hepatocyte with lipid droplet presenting a transition stage Osmiophilic inhomogeneous areas (t) not osmiophilic areas (e) network (n) in the droplet and a capsule like arrangement (c) with small electron dense granules (g) are seen Electron micrograph Stained with zinc uranyl acetate and lead citrate × 36 000

been possible to demonstrate triglyceride between some of the hepatocytes Since it is in 10 μ sections it is not possible to evaluate whether it is placed in bile capillaries sinusoids or perhaps both Dilated intercellular spaces could be seen electron microscopically but demonstrating fat in these intercellulare spaces has not been possible

As mentioned above some of the droplets presented a fine network with capsule like arrangement especially in the not osmiophilic vacuoles At high electron resolution this network appeared very finely granular not lamellar Accordingly it does not seem to represent a remnant of the membranes of the endoplasmic reticulum A meshed network was found by *Camain et al* (1959) in hepatocytes from patients with Kwashiorkor The authors interpreted it to represent shrinked fat in the centre of the droplet Using OTAN staining in the present study this network was stained only very faintly and its morphology in the electron microscope did not indicate fat

Porta et al (1965) described small fat droplets partially surrounded by a limiting membrane possibly derived from smooth surfaced endoplasmic reticulum Such a membrane was not found in the present work but some of the content of the vacuole was arranged like a sort of a capsule

The strongly electron dense granules found both in the triglyceride filled droplets and in the empty ones with network and capsule like arrangement probably correspond to those described by *Jézéquel* (1958) in rat

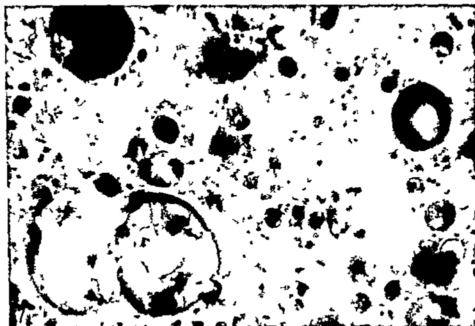


Fig 7 Liver parenchym cells with fat droplets 10 μ thick cryostate section stained with Oil Red O Note the inhomogeneous staining × 500

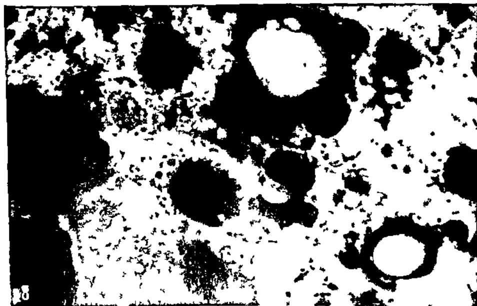


Fig 8 10 μ thick cry s sta ned i l OTAN (osmium tetroxide alfa naphtyl) The triglyceride is stained black The e l ollow black rigl cer de conta n ng droplet is only faintly stained × 500

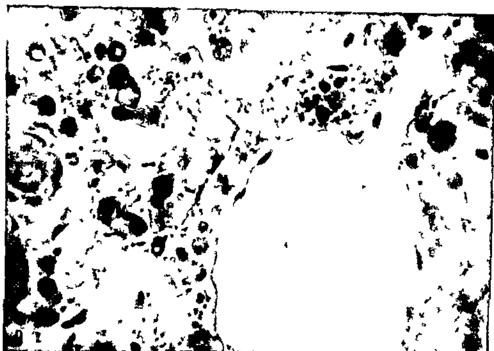


Fig 9 10 μ thick cryostat section stained with Oil Red O Note the fat in the intercellular space × 500

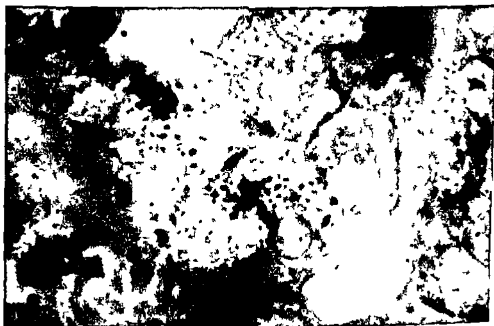


Fig 10 10 μ thick cryostat section stained with OTAN (osmium tetroxide alfa naphtyl) The triglyceride is stained black Note the triglyceride in the intercellular space × 500

livers after intoxication with phosphorous, and by *Camain et al* (1959) in livers from patients with Kwashiorkor They found granules of 60 Å of size and considered them to be ferritin In the present study they presented a varied morphology, some were round some oblong and some were rather pointed and larger than those described by *Je.équel* and *Camain et al* They do not have the characteristics of ferritin granules, and further examinations are necessary to elucidate the nature of these structures

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This study was supported by grants from Statens lægevidenskabelige Forskningsråd and *Købmand i Odense Johann og Hanne Weimann, f Seedorffs legat*

The study was performed at the Institute for General Zoology Copenhagen I wish to thank dr phil *K J Pedersen* for providing these facilities and for advice

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Acta path microbiol scand Section A 82, 264–276, 1974

FOCAL AVILLOUS LESIONS IN THE GUINEA-PIG DUODENUM INDUCED BY FASTING

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In adult male guinea pigs exposed to total fasting avillous areas arose in the duodenum, and sometimes in the proximal jejunum, in the course of 12 18 hours The lesions increased in extent during the first 2–3 days, whereupon the condition was stationary until the animals had been fasted for 5–7 days Then the villi started regenerating After 9 days fasting the small intestinal appearance might be completely normal The pathological process by which the avillous surface formed is described after periodic Schiff (PAS) stained whole mounts had been studied in the stereomicroscope and by conventional histological methods It is suggested that the reaction is elicited by the acid gastric juice which is being secreted in spite of lacking stimulation by food The appearances are compared with those which may be found in patients with the Zollinger Ellison syndrome or with duodenal ulcer

Since Doniac and Shiner (8), in 1957, described the avillous surface of the small intestine in idiopathic steatorrhoea, a greatly increasing interest has been taken in the surface structure of the small-intestinal mucosa under normal and abnormal conditions At the outset the morphological appearances described by *Doniach & Shiner* were considered pathognomonic of gluten induced idiopathic steatorrhoea (25) However, complete or partial loss of villi in the small intestine has been demonstrated in a large number of conditions in man as well as in experimental animals Therefore, the general opinion is now that these appearances may be elicited under several different abnormal circumstances (2 5 26, 30) The mechanism responsible for the characteristic flat appearance of the small intestine still remains unelucidated, but several hypotheses have been adduced (6, 8, 22, 27, 30)

The present paper describes a morphological development which may lead to the formation of avillous areas in the small intestine The studies showed that the apical part of the villi may be desquamated in a well defined, rapid pathological process which leaves an epithelialized, wholly or partially avillous surface

At the same time a pathogenetic factor is described which is able to induce formation of an avillous surface structure It was demonstrated that as a reaction to total fasting guinea pigs soon develop avillous areas in the duodenum and proximal jejunum

The stomach and small intestine of the guinea-pigs were studied by conventional histological methods and in the stereomicroscope after periodic acid Schiff (PAS) staining of whole mounts by the method introduced

Received 23 v 73 Accepted 1 xi 73

by *Landboe Christensen* & *Parapat* in 1970 (16, 17) By this means even minor changes of the mucosal surface structure are visualized and small focal changes may be located

MATERIAL AND METHOD

63 male guinea pigs weighing 600–700 g were divided into 9 groups of 6 or 9 They were fasted in cages with a net floor which prevented coprophagia Water was given *ad libitum* The animals of the 9 groups were fasted for 12 hours 18 hours, 1 2 3, 4 5 7 and 9 days respectively The fasting period was started at times adapted so that all were killed at the same time of the day (about 1 p m)

In addition 9 guinea pigs were used as controls and were sacrificed without any fasting

The sacrifice and preparation were performed as follows The guinea pigs were anaesthetized by intraperitoneal injection of Nembutal (30 mg/kg), and the abdomen was opened The stomach and small intestine were fixed *in situ*, while the blood circulation was still intact by direct intraluminal injection of a 4 per cent formaldehyde solution were cautiously removed cut open and spread out on a polyethylene plate The specimens were stained by periodic acid Schiff (PAS) (16 17) and studied in the stereomicroscope Areas showing changes and control areas were studied histologically after PAS haematoxylin staining

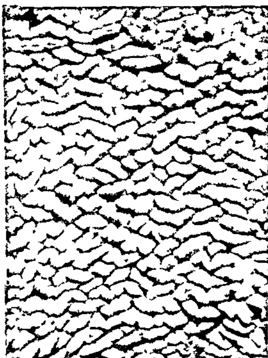


Fig 1 Normal stereomicroscopic surface structure in the guinea pig duodenum Closely packed leaf formed and convoluted villi × 18

RESULTS

It was ascertained that the duodenal mucosa in the guinea pig shows a very strong and characteristic reaction to fasting In the course of less than 24 hours superficial mucosal lesions arose Within the first days they increased in extent and intensity with the duration of fasting Even despite continued fasting however this process was followed by a regenerative phase which was observed in animals killed after having been fasted for 5 days or longer

The lesions were most pronounced in the duodenum decreasing in extent down towards the proximal jejunum in which they were more sporadic Distal to this point there were no changes

In the 9 controls no lesions were found The normal stereomicroscopic picture in the duodenum of the guinea pig showed a few fingerformed villi and else a compound of closely packed leafformed and convoluted villi (Fig 1)

First the degenerative and then the regenerative changes will be described below In each case the stereomicroscopic findings will be reported before the histological ones

I Degenerative Phase

Stereomicroscopic findings In some areas the changes manifested themselves as sharply demarcated lesions in which the villi were enclosed by PAS positive material and in which the villous structure was partially obliterated

In other areas (Fig 2) the morphology was centrally as described above, while in the periphery of the lesions the apical part of all villi had been desquamated the surface consisting merely of transverse ridges representing the common base of the villi They formed long low slightly tortuous rows showing the openings of the crypts as dark, PAS-

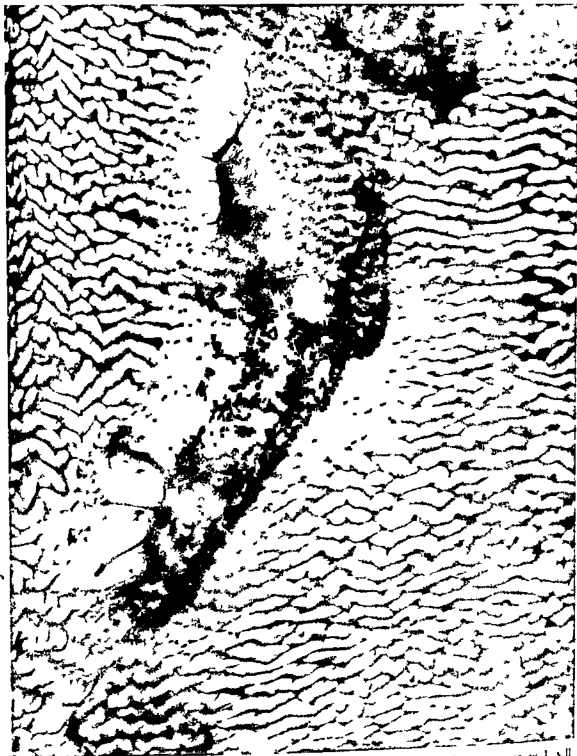


Fig 2 At the bottom of the picture villi enclosed by PAS positive material. Above an area with villi being desquamated. In the periphery "washboard" appearance or completely flat surface. Centrally a layer of mucus, desquamated villous material and exudate. × 15

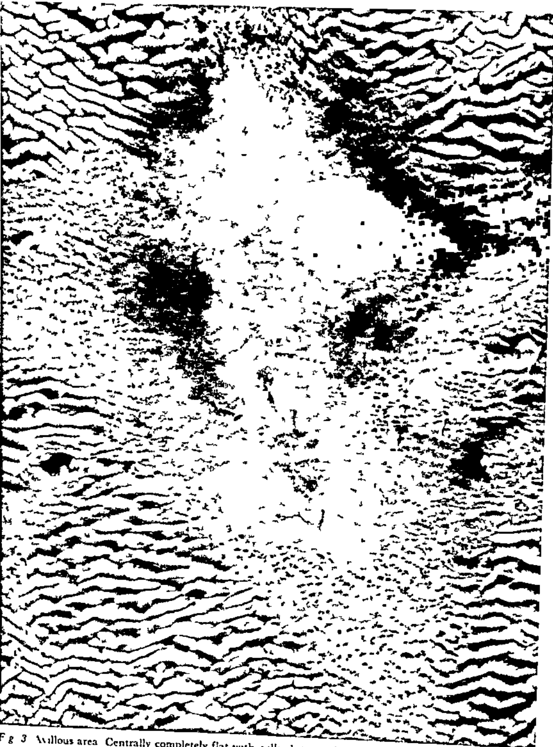


Fig 3 Villous area Centrally completely flat with villi distinguishable In the periphery washboard appearance The crypts are seen as dark dots between the transverse ridges consisting of the common base of villi remaining after the desquamation × 18

8*

positive dots in the interjacent grooves Thus, the surface had a "washboard' appearance

In addition, there were large, sharply demarcated areas in which the apices of the villi were absent throughout (Fig 3) Peripherally in these areas there was the above mentioned "washboard" appearance, while centrally the surface was often entirely avil lous and thus completely flat In a few cases the lesion was so deep that only the crypt epithelium was left as white rings or tubes standing out on a denuded surface

Histological findings In areas where the villi were by way of being desquamated there were in the central part apices demar cated from the base of the villi by a definite constriction at which the villous connective tissue usually displayed an accumulation of polymorphonuclear leukocytes In the apices the epithelial cells were pale, but tall and morphologically normal and connective tissue was scarce (Figs 4–5) In cases where the apices had been entirely detached, the free epithelial edges on the remaining part of the villi were seldom far apart

In the more peripheral part of these areas all apices of the villi were detached, forming a superficial layer of detached apices arranged side by side, surrounded by mucus necrotic cellular material, and exudate in sites where the stereomicroscope had disclosed PAS positive material around the villi Beneath there were low, broad villi, usually covered with an intact, irregular, low cuboidal epithelium staining deeply with haema toxylin (Fig 6)

Centrally in the areas where the process of desquamation had been completed the sur face was usually quite flat, the remaining vital part of the villi being lower than peri pherally (Fig 7) In a few cases the lesion was so deep that only the crypt epithelium remained whereas otherwise the surface was denuded

The inflammatory reaction in the lamina propria after the desquamation was usually slight However, in some cases, frequently where the epithelial defect had not closed there was extensive inflammation which



Fig 4 Histologic section of villus early in the process of desquamation PAS haematoxylin × 400

might involve all layers of the intestinal wall and which might entail accumulations of exudate on the surface

Course of the degenerative process The chronological course of the process may be described on the basis of the small intestinal appearances found in the guinea pigs of each experimental group related to the duration of fasting The size of the altered areas was measured under the stereomicroscope, their approximate area calculated and related to the surface area of the duodenum Changes of the mucosal surface were found in all the guinea pigs studied except those sacrificed after 12 hours fasting The changes observed in the individual groups are tabulated in Table 1 They were as follows

12 hours (6 guinea-pigs) In 3 guinea pigs the duodenal surface showed patches of

Fig 5 Apices of several adjacent villi by way of being desquamated PAS haematoxylin × 125



Fig 6 Process of desquamation nearly finished A superficial layer of detached villi, mucus and exudate has formed PAS haematoxylin × 64

varying size (as a rule about 6–8 mm^2) where the villi were in the process of desquamation The other 3 animals showed no changes

18 hours (6 guinea pigs) All the guinea pigs exhibited the above mentioned lesions Five also showed avillous areas of varying extent and number On the average,



Fig 7 Histologic section of avillous area PAS haematoxylin × 50

TABLE 1 *Number of Guinea-pigs in Each of the Nine Groups With the Mentioned Semimacroscopic Findings in the Duodenum*

No of animals	Time fasted	No changes	Detachment of villi	Avillous areas	Regeneration of villi
6	12 h	3	3	0	0
6	18 h	0	5	5	0
9	1 day	0	8	9	0
6	2 days	0	4	6	0
9	3 days	0	4	9	0
6	4 days	0	1	6	0
9	5 days	0	1	9	5
6	7 days	0	0	6	6
6	9 days	2	0	4	1

about 20–25 per cent of the duodenal surface was involved in the process

24 hours (9 guinea-pigs) The number of areas in which villi were being desquamated had increased The avillous areas made up on the average about 25–30 per cent of the duodenal surface

2 days (6 guinea-pigs) The number of *areas in which the villi were by way of* being desquamated was considerably smaller than in the preceding group The extent of avillous areas averaged about 40 per cent of the duodenal surface

3 days (9 guinea pigs) There were only a few areas in which villi were being desquamated The extent of avillous areas was approximately unchanged

4 days (6 guinea-pigs) Hardly any areas showed desquamation of villi and the extent of avillous areas was still fairly unchanged

The number of areas in which desquamation of villi is taking place reflects the activity of the process at the time of sacrifice, whereas the size of avillous areas indicates the total extent of the degenerative processes which have taken place during the period of fasting It may be said in summary that the degenerative changes ran their course mainly within the first 48 hours fasting, whereas during the next 2 days the process had almost come to standstill, resulting in a *status quo*

In the full blown picture, seen after fasting for 3 and 4 days, the normal duodenal surface was broken by sharply demarcated wide lanes and areas where the *villi were* absent (Fig 8) *In the proximal part of the* duodenum the areas were large and confluent, whereas more distally they *formed* grooves and patches often consecutive, and always oriented in the longitudinal *direction* of the intestine



Fig 8 Full blown p cture (after four days fast ng) PAS sta n ng × 3 5

Regeneratie Phase

Stereom croscopic findings In guinea pigs that had been fasted for 5 days or longer the appearances were different many speci mens showing regeneration of villi in the af fected areas (Fig 9) The earliest sign of such regeneration was more rounded shape of the surface contours the openings of the crypts being depressed in relation to the level of the surface In other sites there might be growth of new villi They might be small thin and of a netlike arrangement but there were also more developed villi almost like those found in the surrounding uninvolved surface The process of regeneration was fairly synchronous within the various areas in the same animal

Histological findings The regenerative changes manifested themselves first by the mucosa increasing in height and at the same time the opening of the crypts being de pressed in relation to the interjacent surface which domed upwards in incipient newfor mation of villi The epithelium which in flat areas had been irregular low cuboidal was during the phase of regeneration tall regular of normal stainability and morphology The new villi which developed were morphologi cally normal except that during the early stages they were small (Fig 10)

There was no or only mild inflammatory reaction in the lamina propria The lamina muscularis mucosae and the submucosa were normal

Regeneration of villi in the small intestine has been studied by int al *Cameron et al* (4)

Course of the regenerative process The chronolog cal relat on was as follows (in addi tion see Table 1)

5 days (9 guinea pigs) The extent of the avillous areas corresponded to the findings at 4 days but 5 animals showed signs of incipient regenerat on of villi in one or more areas

7 days (6 guinea pigs) All the guinea pigs showed signs of regeneration and in 3 animals no completely flat areas remained

9 days (6 guinea pigs) None of the animals exhibited entirely avillous areas In 2 the duodenum was completely normal showing a uniform villous structure throughout and in the remaining animals the process of re generation was far advanced

In addition to the usual lesions 3 guinea pigs had a fullblown acute duodenal ulcer with penetration of the lamina muscularis mucosae Two of these guinea pigs had fasted for 5 days and the third one for 9 days The

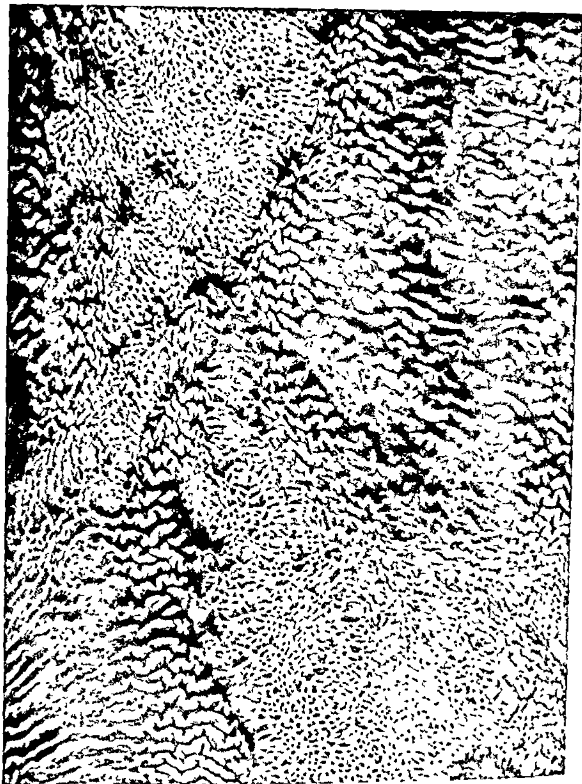


Fig 9 Area with villi in regeneration PAS staining × 18

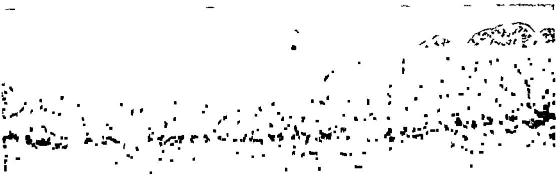


Fig 10 Histologic section of regenerating villi PAS haematoxylin × 64

three ulcers were situated quite proximally in the duodenum Another three guinea pigs had pyloric ulcer, situated in all cases close to the pyloro-duodenal junction Moreover, 13 guinea pigs had one or more minor erosions in the body of the stomach The earliest appearance of such erosions was after 2 days' fasting

DISCUSSION

Several investigators have reported on the effect of fasting upon the gastrointestinal canal in experimental animals (13, 14, 18 20 21, 23) As a rule the experiments have been done on mice or rats which often have been partially fasted or else essential nutritives have been withheld

The pathological findings have been varying and often slight Frequently has been described haemorrages and erosions in the stomach (14, 20, 21 23), and in some cases haemorrhages in the small intestine too (14 20) Others have found only minor changes such as reduced mitotic rate and reduced number of epithelial cells in crypts and on villi of duodenum (13) or only reduced stainability of the cytoplasma in the epithelial cells in the apices of villi (18)

The pathogenetic factor eliciting the morphological changes found in the present experiments in the proximal part of the guinea-pig small intestine in response to total fasting is presumably the acid secretion produced in the stomach in spite of lacking stimulation by food As the stomach is empty, the secretion accumulates, without essential dilution or neutralization in the pylorus from where it is transported down into the duodenum where gradually it will be neutralized in the alkaline environment as it passes down through the intestine Guinea pigs secrete a relatively large amount of acid during fasting This has been studied by *Watt* (31) After fasting guinea pigs for 15 hours, he found a varying, but rather vigorous fasting acid secretion

It is well known that acid may induce surface lesions in the small intestine *Williams* (33) found that rats developed ulcerations when the small intestinal mucosa was exposed for 2 minutes to 0 2 N HCl Similarly, duodenal ulcerat ons may be induced in most experimental animals by increasing the acid production by histamine or gastrin during total fasting (10 11)

The assumption that the gastric juice is the pathogenetic factor which elicits formation of avillous areas is supported by subsequent experiments by *C Fenger* and the present author These experiments showed that this reaction is almost completely prevented if gastric secretion is inhibited by subcutaneous administration of degraded carrageenan (32)

The earliest sign of the morphological development leading to the formation of avillous areas is constriction of the villus At the same time the epithelium apically to the constriction grows pale, and apparently the con-

nective tissue is drawn down to the base of the villus At the site of the constriction there will be an inflammatory reaction with accumulation of polymorphonuclear leukocytes The junction between the pale epithelium apically to the constriction and the unaffected epithelium basally is very sharp and may be defined with an accuracy of almost one cell (Fig 5)

Later, the apical part of the villus is detached, and on top of the remaining part of the mucosa there forms a layer of mucus, desquamated apices lying side by side, and an exudate released from the site of constriction at the desquamation Only very little connective tissue is present in this layer

The formation of a constriction on the villus as well as the drawing down of the connective tissue to its base might be imagined to be due to contraction in the smooth muscles centrally in the villus However, this can probably not be the initial part of the process, as the epithelium on the apex has to be detached before the connective tissue can be drawn down

How, this process is elicited, and what is its initial link is unknown It might be detachment of the epithelium on the apical part of the villus, leading to compromised nutrition of the epithelium and consequent degeneration This again might let acid into the lamina propria and possibly elicit the mentioned contraction in the smooth muscles It might be imagined also that the primary factor was a vascular reaction compromising the blood supply to the apical part of the villus, leading to degeneration and detachment of the epithelium It is difficult to find an explanation affording a satisfactory solution as to why the line of demarcation on the villus is so sharp

Owing to the constriction process the free ends of epithelium on the remaining part of the villus have approached each other, and after the desquamation of the apical part of the villus they rapidly join so that the epithelial cover is defective for only a very short period

The reaction described is relatively atraumatic, because little connective tissue is lost, and generally it does not result in epithelial defects which might cause inflammation as well as ulceration of the mucosa At the same time, it leaves a surface which is able to regenerate soon after the pathogenic effect has ceased Moreover, it may be said perhaps that the reaction is expedient, as it enables the mucosa to reduce its surface area locally, rapidly and without much loss of substance when it is exposed to a pathogenic action which otherwise might possibly lead to inflammation, necrosis, and formation of ulcerations

As stated above, a complete regeneration of the villi sets in after 5–7 days fasting even though the fasting is continued As the villi may regenerate very quickly (4), it must be imagined that the action which elicits the reaction apparently persists for 4–5 days and then subsides, so that the process of regeneration can set in If the HCl pepsin production of the stomach is the eliciting factor, the explanation might be that when for a few days the stomach has not been stimulated by food, the basal acid secretion is reduced, so that the pH in the duodenum rises to the extent of eliminating the tissue damaging effect This accords with the finding of *Robert et al* (23), viz that in the first 2–3 days of total fasting the acid production in rats only changes slightly whereas between the 3rd and 7th day it shows a marked fall

As a curiosity, it may be mentioned that according to a subsequent experiment by the present author the regenerative processes within 9 days fasting may be partially prevented if, instead of being totally fasted the guinea pigs are allowed to nibble at a carrot for one hour every third day By this slight food stimulation then it is possible to maintain the action which brings on the reaction

In relation to the present morphological changes in the small intestine of guinea pigs and the aetiological as well as the pathogenetic contemplations, it may be mentioned that in human pathology too there have been cases in which duodenal biopsies have shown patches of villous atrophy, and this has been ascribed to an increased action by acid upon

the mucosa In several patients with the Zollinger Ellison syndrome short, plump villi or patches devoid of villi have been found in the duodenum and proximal jejunum (7, 28, 29) *Singleton et al* (29), measuring the pH in the duodenum of such a patient found it to vary from 1 5 to 5 5 as compared to the normal approx 7 0 (24) *Shimoda et al* (28) investigated the variations in pH through the intestine in relation to the site of avillous patches in a Zollinger Ellison patient with avillous patches in the duodenum and proximal jejunum Such patches were found to occur exclusively in areas where the pH was below 2 whereas in areas where it exceeded 3 6 the mucosa was always normal In the histological description of the areas he mentioned patches of complete or partial villous atrophy acute inflammation of the lamina propria and at times in the deeper layers superficial microerosions and in some biopsies necrosis of the epithelium in the apices of the villi The changes disappeared after total gastrectomy

Patients with duodenal ulcers too may exhibit areas of the duodenum which are wholly or partially avillous This was found by *Aronson* & *Norfleet* (3) in 4 out of 18 patients with duodenal ulcer According to *James* (15), this is common in patients with duodenal ulcer less common in pyloric ulcer and rare in cases of ulcer in the body or fundus of the stomach

It is common to patients with duodenal ulcer and patients with the Zollinger Ellison syndrome that both have a high acid production in particular a high fasting and basal acid production (9 10 19) The acid exerts its tissue damaging effect upon the mucous membrane mainly during the night when the stomach and duodenum do not contain food and when these patients cannot reduce the acid production to the same extent as other people

Dragstedt (9 10) has pointed out the diluting and neutralizing effect of food upon the gastric secretion and the equilibrium between the amount of secretion and the food as the most important factors in preventing the occurrence of peptic ulcerations

Regarding the rapid formation of the avillous lesions in the present work it can be mentioned that this too is well known in the human pathology Thus it was demonstrated in a child with an soy protein intolerance that feeding soy protein caused reversible, flat intestinal lesion in less than 12 hr (1)

Considering the above description of the morphological changes which may be observed in the small intestine of patients with the Zollinger Ellison syndrome or with duodenal ulcer, it is not unreasonable to imagine that the pathological reaction occurring in the guinea pig duodenum and proximal jejunum after brief fasting may be observed also in man elicited locally when the pH in the small intestine falls below a certain level

At present it cannot be ascertained whether this reaction is possibly involved in the formation of the avillous small intestinal surface seen in the presence of other disease states than the ones described above and whether it may be elicited by pathogenic factors other than a low pH The process which makes small intestinal villi apparently disappear in connection with various states of disease (e g gluten induced enteropathy) is still unknown The reaction described in the present paper was observed in a relatively large material in which all developmental stages appear to be represented and under experimental conditions which are not unphysiological Therefore it is a possibility which should probably be included in the contemplations when discussing the pathogenesis of avillous areas in the small intestine

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LOCALIZATION AND PERSISTENCE OF COMMON ENTEROBACTERIAL ANTIGEN AND TYPE-SPECIFIC BACTERIAL ANTIGEN IN EXPERIMENTAL PYELONEPHRITIS

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A strain of *E coli* (04 U4/41) was used to produce retrograde pyelonephritis in rats By means of indirect immunofluorescence technique the localization and persistence of common enterobacterial antigen (CA) and type specific bacterial antigen (TSA) in the inflamed kidney tissue were followed for 4 months It appeared that demonstrable bacterial antigen disappeared from the tissue during this period In amorphous form, CA and TSA were initially found in the same localizations but did not show parallelism in persistence, as CA in nearly all localizations disappeared earlier from the tissue than TSA this could be observed especially in macrophages

Common enterobacterial antigen (CA), described by *Kunin* in 1963 (7), has been demonstrated in kidney tissue with experimental pyelonephritis by means of immunofluorescence technique by *Aoki et al* (1) and *Miller et al* (8), and in 1969 *Aoki et al* (2) found CA also in human kidney tissue with chronic pyelonephritis, viz in 6 cases of so called 'bacterial' chronic pyelonephritis, and in 6 cases of chronic pyelonephritis without demonstrable viable bacteria in the urine or kidney tissue at the time of examination

In a previous study (12) it was shown that CA could be demonstrated by means of immunofluorescence technique in kidney tissue with experimental haematogeneous pyelonephritis produced by various strains of *E coli* However, in the 6 cases showing chronic inflammatory changes, no CA was revealed, [illegible]

than TSA, especially in macrophages However, the haematogenous path of infection which was used, appeared to be less suitable for accurate evaluation of the localization and persistence of the two forms of antigen as this method very easily gave rise to massive abscesses and destructive kidney lesions, often resulting in pyonephrosis Accordingly, it was difficult to produce a sufficiently large number of kidneys with lesions comparable to those seen in human chronic pyelonephritis

It was therefore found desirable to make an attempt to throw light on this apparent lack of parallelism between CA and TSA in their occurrence in the inflamed kidney tissue by following the two forms of antigen in their pathological localizations and persistence in

Received 30 x 73 Accepted 30 x 73

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a number of kidneys exhibiting a gradual change from acute to chronic inflammation With this end in view, a retrograde route of infection was chosen, as it seemed superior to the haematogeneous path in producing a form of inflammation which was suitable for these observations

For the infection we selected *E coli* 04 U4/41, which in the previous study had proved to be suitably virulent and to contain CA

Clear differences in persistence but not in localization, between the two forms of antigen were observed in the study described below

MATERIAL AND METHODS

Experimental procedure Experimental retrograde pyelonephritis was produced in white female Wistar rats weighing 180–200 gm The animals were fed laboratory chow and water *ad libitum* The method described by *Heptinstall* (6) was used in a slightly modified form Under intraperitoneal light pentobarbiturate anaesthesia the urinary bladder was palpated, and filled bladders were emptied by gentle compression through the abdominal wall Then the bacterial suspension was introduced into the bladder from a 2 ml syringe *via* a catheter (the cut proximal polyethylene part of a BARDIC I catheter originally intended for transduction through a 19 gauge needle) which was inserted into the urethra An amount of 0 5–0 6 ml of the suspension was injected after which the bladder was gently squeezed about 20 times through the abdominal wall During this the external orifice of the urethra was compressed between two fingers in order to prevent the suspension from escaping It has been shown that this procedure causes reflux into the ureters and the renal pelvis (*Heptinstall* 6)

Bacteria The injected bacterial strain was *E coli* 04 U4/41 A suspension containing approx 1×10^9 viable organisms per ml was prepared as previously described (12)

Sampling of kidney tissue At different time intervals after the infection animals were killed by heart puncture exsanguination under intraperitoneal pentobarbiturate anaesthesia About one third of the infected kidney was removed aseptically for bacterial culture primarily in order to ascertain that the cultured bacteria were identical with the infecting strain this was carried out by bacterial agglutination using the homologous antiserum The remaining kidney tissue was quick frozen in gelatine some drops of which were placed on a copper block precooled to $-70°$ C at which temperature the gelatine embedded tissue was stored until required The tissue blocks were sectioned in a cryostat at $-20°$ C Consecutive sections 4 6 μ in thickness were placed on cleaned slides, fixed in acetone for 10 minutes and then dried at room temperature for 30 minutes before staining In many cases paraffin blocks of kidney tissue were also prepared according to the method of *Sainte Marie* (9) Sections of tissue prepared by these methods were used partly for light microscopic examination after staining with haematoxylin eosin periodic acid Schiff (PAS), and Sirius stain for connective tissue and partly for immunofluorescence studies for which both methods were suitable

Immunofluorescence staining The staining procedure was carried out as previously described (12) The sections were stained by the indirect immunofluorescence technique, the first layer was an unlabelled rabbit antiserum against bacterial antigen—either anti CA or anti *E coli* 04—and the second layer a commercially available fluorescein isothiocyanate labelled antiserum against rabbit immunoglobulin (FITC labelled goat anti rabbit γ globulin Behringwerke)

Testing of the FITC labelled antiserum by immunoelectrophoresis revealed a single strong precipitation line at the site of rabbit IgG

Antisera Antisera against *E coli* 014 (anti-CA serum) and against *E coli* 04 (anti-04 serum) were produced in rabbits and tested as previously described (12) Moreover reference 0 antisera against *E coli* 014 and *E coli* 04 were used in some investigations (These antisera as well as the test strains of *E coli* 014 and *E coli* 04 were kindly supplied by Dr F Ørskov The International Escherichia Centre Statens Seruminstitut Copenhagen)

Microscopic examination A Zeiss universal fluorescence microscope was used with optical conditions as previously described (12 To facilitate comparison consecutive sections were stained with anti CA serum and anti 04 serum As far as possible the same fields as they occurred in the consecutive sections, were observed and photographed

Specificity controls Such controls were carried out as previously described (12) Fluorescent structures were compared with exactly the same structures in the consecutive control sections Only if fluorescence in these was totally absent or at least definitely weaker than that of the sections stained with antiserum was it considered to be specific

Stainings were carried out with and without the initial fixation of the cryostat sections for 10 minutes in acetone in order to examine whether this procedure had any influence on the persistence in the tissue of the fluorescent structures this was not the case The acetone fixed sections yielded a better morphological presentation of the tissue than the unfixed ones

RESULTS

Bacteria were injected into the urinary bladder of 104 rats Of these, 34 died less than 24 hours after the injection, usually without having emerged from the anaestesia Five died during the period of examination Of the 65 animals surviving the operation, 55 showed signs of gross and microscopic pyelonephritis in one or both kidneys Four rats had pyelitis, and one had pyonephrosis as the only lesion In five rats, neither gross nor microscopic lesions in the kidneys were seen A survey over the kidney lesions distributed on the number of rats is given in Table 1

The described retrograde route of infection generally resulted in an inflammatory pattern, as illustrated in the diagram in Fig 1 Initially, haemorrhages were seen in the forniceal regions which are considered to be the port of entry of the infection Inflammatory lesions involved these areas, as well as the medial anterior or posterior part of the kidney, and the peripheral, proximal half of the papilla In contrast, the lateral region of the kidney and the central part of the papilla were rarely involved In these relatively well defined areas, inflammatory changes progressed, accompanied by deforming contractions of the tissue

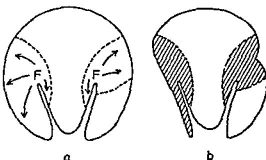


Fig 1 a Transverse section of the rat kidney, illustrating the presumed port of entry and typical extension of the infection (areas within broken lines) F = fornix

b The medial parts of the kidney are the sites of contractions, the chronically inflamed areas are hatched

Gross and Light Microscopic Changes

In the first few hours after the bacterial injection, the kidney was of normal size After 10 minutes fresh haemorrhages were seen in the pelvic lumen and in forniceal areas, presumably because of rupture of small vessels in these areas caused by the pressure transmitted by the injected suspension At 60 minutes, numerous eosinophilic leucocytes were seen as the first inflammatory cells situated in the hilus of the kidney, especially in and around the adventitia of the branches of the renal artery, and beneath the parietal pelvic epithelium At 6 hours, small collections of polymorphonuclear leucocytes also occurred mostly related to the haemorrhagic areas

At 22–24 hours, the kidney was slightly enlarged with small yellowish abscesses, mostly in the medial part of the anterior and/or the posterior surface Microscopically, the haemorrhage was in several cases seen to extend from the forniceal region to the medial cortical surface, with well-developed fresh abscesses related to the haemorrhagic areas Moreover, polymorphonuclears were diffusely scattered in the interstitium and in the lumina of dilated tubules and collecting tubules The renal papilla was not yet involved In the pelvic lumen, large numbers of polymorphonuclears were present

During the next few days, the kidney was enlarged, at the 4th day, it was about twice its normal size, after which it diminished again On the surface, yellowish partly confluent infiltrates were seen, which gradually flattened Microscopically, the inflammatory changes now also involved the peripheral proximal half of the papilla Usually, the pelvic epithelium was not, or only slightly, inflamed, whereas polymorphonuclears were seen beneath the pelvic visceral epithelium and in and around the dilated collecting tubules Interstitial mononuclear cells with PAS positive cytoplasm were observed, especially around the abscesses and at the fornix Lymphocytes and mononuclear macrophage like cells were seen from the 3rd day, mostly in the cortex

At the 7th day, both extensive infiltrates of

lymphocytes and scattered plasma cells were common newly formed connective tissue was seen around the abscesses, and the epithelial cells of many tubules and collecting tubules were swollen The glomeruli were intact even in areas with heavy inflammation

During the 2nd week flattened, yellowish white infiltrates were predominant, and incipient cortical contractions were seen Microscopically, many interstitial lymphocytes and macrophages, often in wedge shaped formations, were seen, the amount of connective tissue was increased The inflamed cortical areas showed pronounced tubular loss yet with some tubules dilated and filled with debris and inflammatory cells Beneath the parietal pelvic epithelium many lympho cytes were seen These lesions progressed during the 3rd and 4th weeks, with a gradually diminishing number of polymorphonuclears

During the 2nd, 3rd and 4th months the cortical contractions increased, and the size of the kidney decreased concurrently with the severity of the contractions The contracted areas were characterized by connective tissue and closely packed glomeruli often with a thickened Bowmann capsule, but without sclerosis Infiltrates of lymphocytes were seen to varying degrees, and haemosiderin containing cells were encountered In many cases the medial areas were now reduced to quite narrow edges of tissue The vessels were unchanged Dilated tubules were now seen mainly in the forniceal regions Fibrously encapsulated abscesses persisted in a few kidneys

Immunofluorescence Examination

The bacterial antigen appeared with a bright green specific fluorescence in two different forms (1) as whole, recognizable organisms and (2) as amorphous material In the tissue bacterial antigen was exclusively seen in inflamed areas not in adjacent non

TABLE 1 *Kidney Lesions the Number of Animals with Demonstrable CA and TSA and the Number of Animals with Positive Bacterial Growth among 65 rats*

Period	No of animals	Kidney lesions	No	CA Whole bacteria	CA Amorphous bacterial antigen	TSA Whole bacteria	TSA Amorphous bacterial antigen	Post bacter cultur
0 24 hours	7	Forniceal haemorrhage	2	2	0	2	0	2
		Pyelonephritis	4	4	3	4	3	4
		Pyelitis	1	1	0	1	0	1
[illegible] days	18	Pyelonephritis	15	14	15	14	15	14
		Pyelitis	2	0	0	0	0	1
		No lesions	1	0	0	0	0	1
8 14 days	7	Pyelonephritis	7	5	7	5	7	7
15 28 days	11	Pyelonephritis	9	7	7	6	9	9
		No lesions	2	0	0	0	0	0
2nd month	9	Pyelonephritis	7	4	6	4	8	7
		Pyonephrosis	1	1	1	1	1	1
		No lesions	1	0	0	0	0	0
3rd month	7	Pyelonephritis	6	2	2	3	3	4
		No lesions	1	0	0	0	0	0
4th month	6	Pyelonephritis	5	1	0	1	3	3
		Pyelitis	1	0	0	0	0	1

inflamed regions of the kidney Bacterial antigen visualized by means of anti 04 serum and anti CA serum is denoted as TSA and CA, respectively Table 1 shows, compared with the results of bacterial culture, the number of animals with the two forms of antigen demonstrated in one or both kidneys, and Fig 2 gives a survey of the observed persistence in the different localizations

As to the demonstration of bacterial anti gen, no differences in the results were ob served between our antisera and the referen ces 0 antisera

Whole Bacteria

During the first few days, TSA and CA in the form of recognizable bacteria were seen with similar intensity of fluorescence, after some days, many bacteria were seen with stronger fluorescence for TSA than for CA, however, without a difference in the persist ence in time After 10 and 60 minutes, the bacteria were seen in the *pelvic lumen* and *interst tially*, related to the forniceal haemorrhage, following this into the renal parenchyma At 6 hours, they were found, partly in clumps, in the *developing abscesses*, and in the *tubules and collecting tubules* from the fornix to the cortical surface The bacteria might be seen in clumps adhering to the wall of the tubule (Fig 3) and some were seen intracellularly in the *epithelial cells* At about 48 hours, they were present in the *collecting tubules of the papilla*, peripherally in its proximal half Occasionally, bacteria could be seen clumping around and phagocytized by interstitial *macrophages* during the 1st week During the first few days, bacteria were seen in large numbers, but they could be demonstrated in decreasing numbers in some kidneys up to nearly 4 months, persisting for the longest time in encapsulated abscesses and in the lumina of dilated forniceal tubules

Amorphous Bacterial Antigen

This was seen in the following localiza tions

Abscesses and pelvic lumen Here, TSA and CA were seen with the same persistence and intensity of fluorescence in and around the numerous polymorphonuclears During the 1st week, the occurrence was massive, later with a distinctly weaker fluorescence

Cortical tubules From the 2nd day these were dilated, filled with polymorphonuclears in which TSA and CA could be seen With progressive inflammation, heavy destruction of cortical tubules occurred, in luminal remnants small clumps of bacterial antigen might be seen In these, TSA could be demonstrated for up to 14 weeks, while CA was not ob served later than 4 weeks

Lumina and epithelial cells of tubules/collecting tubules of the fornix and papilla At the fornix, dilated tubules and collecting tubules were seen, in the lumina of which TSA and CA were present in and around polymorphonuclears At about 48 hours, the collecting tubules in the proximal, peripheral part of the papilla were also involved, with a similar occurrence of TSA and CA

The epithelial cells were often fluorescent, indicating the presence of TSA and CA In the epithelial cells TSA might be seen up to the 15th week, CA only for about 4 weeks In dilated forniceal tubules, TSA might be seen in the 15th week, CA in the 11th week, during the last few weeks with less intensity of fluorescence than TSA

Macrophages Here the most marked dif ferences were seen After 2–3 days, TSA and CA occurred with equally strong fluorescence in numerous macrophages (Fig 5) These were seen in the area from the fornix to the cortical surface, often in wedge-shaped streaks, and in smaller numbers in the inflamed part of the papilla However, as early as the 4th day the macrophages exhibited weaker fluorescence for CA than for TSA, and by the end of the 1st week, TSA was seen, partly with stronger fluorescence, partly in more macrophages than CA, indicating that at this point of time macrophages were present, which contained TSA, but not—or only to lesser degrees—CA (Fig 6 and 7) This tendency continued henceforth During the 2nd week, still fewer and weaker fluores cent CA containing macrophages were seen,

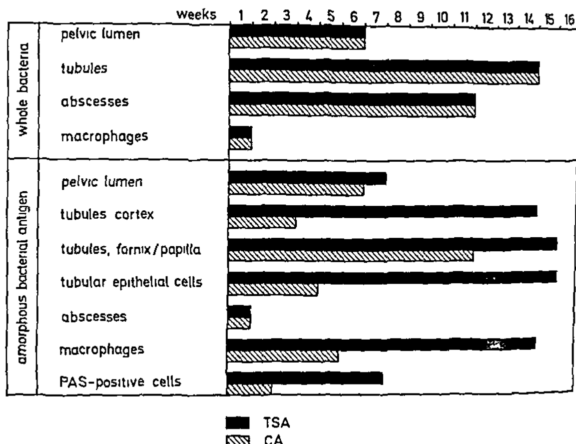


Fig 2 Main localizations and observed persistence of TSA and CA By "persistence" is understood the last point of time at which bacterial antigen, even in minute amounts, was observed in the localization in question However, it should be noted that the figure does not illustrate the *gradual* disappearance of bacterial antigen in the individual localizations, for example, the distinct decrease in the number of CA containing macrophages which was observed as early as the 2nd week

by the end of the 4th week these cells occurred only sporadically and were weakly fluorescent, they were seen up to the 5th week in the forniceal region In contrast, the numerous TSA-containing macrophages exhibited unchanged strong fluorescence until the beginning of the 2nd month, when an increasing autofluorescence began, in the form of yellow clumps and granules, concurrently with which gradually fewer specifically fluorescent TSA-containing macrophages were seen, these were found until the 14th week, also in the forniceal region

PAS-positive cells Mostly localized around abscesses and at the fornix, these cells were seen from about the third day with similar fluorescence for TSA and CA At the 2nd week, intracellular yellow autofluorescence began to manifest itself However, the cells could still be seen containing specifically fluorescent bacterial antigen, now stronger for TSA than for CA During the 2nd week the intracellular content of CA decreased, whereas TSA could still be demonstrated up to 7th week From this time onwards, only autofluorescence was seen in the cells

Interstitium By far the greatest part of the bacterial antigen in the interstitium seemed to occur intracellularly in macrophages, only to a slight degree could it be seen without definite relation to cells

Pelvic epithelium Only in one single case,

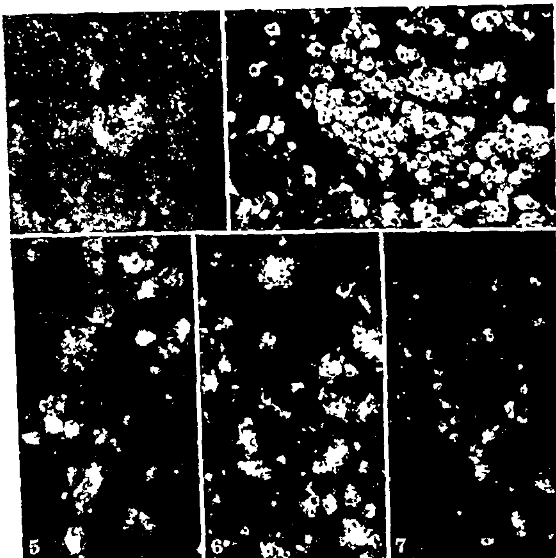


Fig 3 Cryostat section stained with anti 04 serum Whole bacteria are seen in the tubular lumen and adhering to the wall, partly in clumps, 24th hour of infection × 300

Fig 4 Paraffin section stained with anti 04 serum Numerous macrophages as they present themselves in a paraffin section prepared according to the Sainte-Marie method 8th day of infection × 300

Fig 5 Cryostat section stained with anti CA serum Macrophages with bright cytoplasmic fluorescence, 3rd day of infection × 300

Fig 6 Cryostat section stained with anti 04 serum Macrophages with bright cytoplasmic fluorescence, 6th day of infection × 300

Fig 7 Cryostat section stained with anti CA serum Same area as in Fig 6 in a consecutive section On the 6th day, macrophages stained for CA are distinctly weaker fluorescent, both compared with those stained for TSA (Fig 6) and with those stained for OA on the 3rd day (Fig 5) × 300

with a localized necrotic ulceration, was bacterial antigen observed in the pelvic epithelium

Neither whole bacteria, nor amorphous bacterial antigen was observed in the *glomeruli* or *vessels* nor in relation to *lymphocytic infiltrates*

The paraffin sections, prepared according to the method of *Sainte Marie*, yielded the same immunofluorescence results as the *cryostat sections*, however, the morphological picture was generally better in the former ones (Fig 4)

Control sections Specific fluorescence was not observed in the control sections Autofluorescence in these was of the same appearance as in the specifically stained sections

Growth results Tissue for culture was removed from the kidneys in order to ascertain whether they contained viable bacteria, and to see if the cultured bacteria were identical with the infecting strain of *E coli* The latter was always the case, but in a few cases a slight contamination with staphylococci and Proteus was observed In a semi quantitative evaluation massive growth mostly occurred during the first seven days after which time moderate or slight growth was usual Bacteria could be cultured from kidneys during almost the whole period of investigation (Table 1) Positive growth and negative immunofluorescence findings were met in a total of six kidneys negative growth and positive immunofluorescence findings in 15 kidneys, including seven in which both whole bacteria and amorphous bacterial antigen were present whereas only amorphous antigen was seen in the remaining eight

DISCUSSION

The retrograde route of infection fairly easily led to a renal inflammation which was essentially identical in the individual animals, involving well defined areas of the kidney tissue This method resulted in both a larger number of kidneys with the desired lesions and a relatively standardized field of inflammation—in contrast to that of haematogenous infections—which was considered to be of importance in this study

The aforementioned morphological changes correspond fairly closely to those reported in *Heptinstall's* study of the ascending infection in rats (6) Very characteristic is the typical involvement of the medial areas with fairly well defined borders to non inflamed kidney tissue, in contrast to the disseminated abscesses of the haematogenous infection involving the greater part of the kidney tissue The right and the left kidney were approximately equally involved without significant differences as to type of lesion or number of infected kidneys thus indicating a fairly stable mode of infection

The initial typical acute inflammation rapidly progressed into lesions which are characteristic of chronic inflammation (lymphocytes, fibrosis, tubular loss), in many respects resembling the picture of human chronic pyelonephritis However it should be stressed that this study left the impression of a self limiting self healing inflammation rather than an actively progressing type of inflammation in consequence of persisting stimuli in the tissue

As to the *pathogenesis* of the retrogradely induced experimental pyelonephritis it should be noted that we share *Heptinstall's* view of the initial forniceal haemorrhages as an important port of entry of the infection *Heptinstall* expressed the view that the infection begins in relation to the fornix and beneath the epithelium of the pelvis calyx and fornix then spreading partly interstitially following the haemorrhage and partly canalicularly In the present study, the bacteria were initially seen related to the forniceal haemorrhages following these into the parenchyma or into the tubules and collecting tubules passing through this area The involved tubules are connected with the medial parts of the kidney and with the proximal peripheral part of the papilla, which are consequently infected Corresponding to this, the lateral part of kidney is usually free of infection as bacteria in the initial phase generally have not invaded the central papillary tubules con

nected with the lateral kidney areas Undamaged pelvic epithelium did not seem to be of importance as port of entry of the bacteria, as neither bacteria nor essential inflammation was observed here A haematogenous element in the retrograde infection, as postulated by *Tuan et al* (13), seemed less probable because of the well defined inflammatory areas, nor were bacteria in the glomeruli or vessels observed at any point of time

In this study, bacterial antigen in the form of *whole*, recognizable organisms were revealed by using antiserum against CA as well as against TSA however, after some days with stronger fluorescence for TSA, this phenomenon was especially met in bacteria in the pelvic lumen and in dilated collecting tubules and might possibly be caused by a more rapid disappearance of CA than of TSA in dead, but still recognizable organisms in these localizations which were at the same time characterized by containing large numbers of polymorphonuclears No differences were noted regarding the persistence in time of the two forms of antigen in whole bacteria

A different situation obtains with *amorphous* bacterial antigen, where distinct differences in the persistence of TSA and CA were observed, especially evident in connection with the macrophages As early as the 4th day, these cells exhibited weaker fluorescence for CA than for TSA, and during the 2nd week a decrease in the number of CA containing macrophages was noted, which then occurred only sporadically and weakly fluorescent, contrasting to the numerous TSA-containing macrophages which could be seen for several weeks longer than the former ones, until they disappeared concurrently with the increasing autofluorescence The changes in the macrophages leave the impression that bacterial antigen is especially catabolized in these cells, in agreement with this is the observation that bacterial antigen in essential amounts did not seem to occur freely in the interstitium, but predominantly intracellularly in the macrophages

In the interstitial PAS positive cells at the fornix and around abscesses, i e in areas with a massive initial concentration of bacteria, bacterial antigen could be demonstrated at a relatively early stage, during the progressive inflammation these cells developed a clearly intracellular autofluorescence, which after a few weeks was absolute, like *Tan & Heptinstall* (11), we consider these cells to be a form of macrophages

Bacterial antigen could be demonstrated for the longest period in the lumina of forniceal tubules, amorphously in or among polymorphonuclears, sometimes together with free organisms and it is reasonable to assume that bacteria in this localization are able to keep alive and multiply for the longest period

The observed difference in the occurrence of TSA and CA in cortical tubules may be due to the pronounced tubular loss leaving only small remnants of tubular lumina with a content of bacterial antigen, for merely quantitative reasons, this might be thought to favour the demonstration of TSA The relatively early disappearance of bacterial antigen in abscesses and in the pelvic lumen might be due to the catabolic function of the polymorphonuclears

The immunofluorescence study leaves, on the whole, the impression that bacterial antigen in experimental pyelonephritis does not remain without reaction in the tissue, but is to a high degree altered—destroyed, decomposed or neutralized by antibodies CA apparantly sooner than TSA—so that bacterial antigen after some time is unable to react with antisera directed against it

As mentioned this study has shown persistence of TSA for nearly 4 months In this connection, it should be mentioned that *Sanford et al* (10) demonstrated amorphous *E coli* antigen in haematogenous pyelonephritis for 10 weeks, and that *Cotran et al* (5), in retrograde pyelonephritis, demonstrated amorphous *E coli* antigen for 14 weeks, whereas Proteus antigen could be demonstrated for 13 months in retrograde pyelonephritis (3), however, only for 20 weeks

after antibiotic treatment (4) These investigators used rats as experimental animals and type specific antisera in the immunofluorescence investigation

This study has confirmed our suggestion of a lack of parallelism in the occurrence of demonstrable TSA and CA in kidney tissue with experimental pyelonephritis However, nothing definite can be said of the reason why CA is apparently decomposed more rapidly than TSA The lipopolysaccharide part in the bacterial wall is thought to contain two antigenic determinants, CA and O antigen, which presumably exist as a complex Alterations in the lipopolysaccharide molecule during the inflammation might be thought to loosen this complex and somehow expose CA to a more rapid decomposition, possibly by means of antibodies, a search for circulating antibodies might help to clarify this

As to the applicability of the method to human kidney tissue with chronic pyelonephritis, these results do not give rise to increased optimism, provided of course that *corresponding conditions are met in human* tissue, as much evidence indicates that CA must be relatively fresh in order to be demonstrated in the kidney tissue by the immunofluorescence technique

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THE *IN VITRO* MATURATION OF THE EMBRYONIC CHICKEN THYMUS

3 *Quantitative Studies of the Lymphoid Development in Organ Culture*

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The thymic anlagen of 10 day old chicken embryos showed a reproducible development in organ culture, assayed as the number of lymphoid cells obtained per anlage The time dependent increase of the number of lymphoid cells exhibited an exponential phase during culture on days 0–6 followed by a plateau phase during days 8 12 The characteristic growth curve was not significantly changed by subcultivation of whole or divided thymic anlagen or medium changes, in order to improve the culture conditions The lymphoid development of the thymic anlagen in organ culture required chicken serum as medium supplement The number of lymphoid cells per anlage showed an approximately linear relation to the logarithm of the serum concentration within a range of 2 5–20 per cent serum Additional and removal of organ culture medium serum supplement indicated that serum was necessary both for the initiation and maintenance of the thymic lymphoid development

Several factors determine the lymphopoietic activity of the thymus Firstly, a continuous inflow of bloodborne lymphoid precursor cells into this organ takes place both during foetal and adult life (7, 14, 16, 18) Secondly, extensive proliferation and differentiation of lymphocytes occur within the thymus During this process, lymphocytes acquire new morphologic (13), antigenic (17) and functional (17, 19, 20) characteristics. This process may be determined both by the thymic microenvironment itself and external factors acting on the thymus (13, 14) Thirdly, emigration of thymic lymphocytes to peripheral lymphoid tissue (5, 11) may be of significance, particularly during steady state lymphopoiesis

Received 30.x 73 Accepted 27 xi 73

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The essential immunological role of the thymus (4, 15) motivates more extensive investigations of the development of lymphocytes in this organ Such studies are necessarily complicated by the dynamic nature of thymic lymphopoiesis Thus there is not only an influx of lymphoid precursor cells into the thymus and an efflux of thymic lymphocytes, but also a significant traffic of cells from the bursa of Fabricius (8, 9) and possibly of peripheral blood lymphocytes (6) to the thymus of the chicken It follows that an experimental model, where this traffic of cells to and from the thymus could be controlled, would be desirable

Thymic organ cultures offer this advantage They provide the opportunity to study thymic lymphopoiesis uninfluenced by cell traffic and to examine potential thymus growth promoting and inhibiting factors in a direct way

after antibiotic treatment (4) These investigators used rats as experimental animals and type-specific antisera in the immunofluorescence investigation

This study has confirmed our suggestion of a lack of parallelism in the occurrence of demonstrable TSA and CA in kidney tissue with experimental pyelonephritis However, nothing definite can be said of the reason why CA is apparently decomposed more rapidly than TSA The lipopolysaccharide part in the bacterial wall is thought to contain two antigenic determinants, CA and O-antigen, which presumably exist as a complex Alterations in the lipopolysaccharide molecule during the inflammation might be thought to loosen this complex and somehow expose CA to a more rapid decomposition, possibly by means of antibodies, a search for circulating antibodies might help to clarify this

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The thymic anlagen of 10 day old chicken embryos showed a reproducible development in organ culture, assayed as the number of lymphoid cells obtained per anlage The time dependent increase of the number of lymphoid cells exhibited an exponential phase during culture on days 0–6 followed by a plateau phase during days 8–12 The characteristic growth curve was not significantly changed by subcultivation of whole or divided thymic anlagen or medium changes in order to improve the culture conditions The lymphoid development of the thymic anlagen in organ culture required chicken serum as medium supplement The number of lymphoid cells per anlage showed an approximately linear relation to the logarithm of the serum concentration within a range of 2 5–20 per cent serum Additional and removal of organ culture medium serum supplement indicated that serum was necessary both for the initiation and maintenance of the thymic lymphoid development

Several factors determine the lymphopoietic activity of the thymus Firstly, a continuous inflow of bloodborne lymphoid precursor cells into this organ takes place both during foetal and adult life (7, 14, 16, 18) Secondly extensive proliferation and differentiation of lymphocytes occur within the thymus During this process, lymphocytes acquire new morphologic (13) antigenic (17) and functional (17, 19, 20) characteristics This process may be determined both by the thymic micro environment itself and external factors acting on the thymus (13, 14) Thirdly, emigration of thymic lymphocytes to peripheral lymphoid tissue (5 11) may be of significance particularly during steady state lymphopoiesis

Received 30 x 73 Accepted 27 xi 73

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The essential immunological role of the thymus (4, 15) motivates more extensive investigations of the development of lymphocytes in this organ Such studies are necessarily complicated by the dynamic nature of thymic lymphopoiesis Thus there is not only an influx of lymphoid precursor cells into the thymus and an efflux of thymic lymphocytes, but also a significant traffic of cells from the *bursa of Fabricius* (8, 9) and possibly of peripheral blood lymphocytes (6) to the thymus of the chicken It follows that an experimental model, where this traffic of cells to and from the thymus could be controlled, would be desirable

Thymic organ cultures offer this advantage They provide the opportunity to study thymic lymphopoiesis uninfluenced by cell traffic and to examine potential thymus growth promoting and inhibiting factors in a direct way

after antibiotic treatment (4) These investigators used rats as experimental animals and type-specific antisera in the immunofluorescence investigation

This study has confirmed our suggestion of a lack of parallelism in the occurrence of demonstrable TSA and CA in kidney tissue with experimental pyelonephritis However, nothing definite can be said of the reason why CA is apparently decomposed more rapidly than TSA The lipopolysaccharide part in the bacterial wall is thought to contain two antigenic determinants, CA and O antigen, which presumably exist as a complex Alterations in the lipopolysaccharide molecule during the inflammation might be thought to loosen this complex and somehow expose CA to a more rapid decomposition, possibly by means of antibodies, a search for circulating antibodies might help to clarify this

As to the applicability of the method to human kidney tissue with chronic pyelonephritis, these results do not give rise to increased optimism, provided of course that corresponding conditions are met in human tissue, as much evidence indicates that CA must be relatively fresh in order to be demonstrated in the kidney tissue by the immunofluorescence technique

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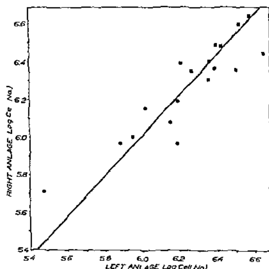


Fig 1 Lymphoid cell yield (Log [No cells/anlage]) of the right and left thymic anlagen of 10 day old chick embryos after 6 days in organ culture Each point represents one embryo First experiment (●) second experiment (■)

left thymic anlage Furthermore, no significant difference ($t = 1.11$, $P > 0.05$) between the number of lymphoid cells of the right and left anlage was noted as indicated by the slope (1 04) of the regression line

The mean lymphoid cell yield per anlage in the two experiments documented in Fig 1 were $1.24 \pm 0.58 \times 10^6$ and $2.82 \pm 0.80 \times 10^6$ (log values 6.02 ± 0.24 and 6.44 ± 0.11)

In the last 24 experiments performed in the present series of investigations, where the time of storage at 12° C of the fertilized eggs was controlled and not exceeded 7 days, the mean cell yield per thymic anlage after 6 days in culture was $3.50 \pm 0.90 \times 10^6$ (log value 6.54 ± 0.13)

Time dependent development of the thymus The time dependent change in the number of lymphoid cells per thymic anlage was studied Organ cultures of thymic anlagen of 10 day old chick embryos were initiated The mean number of lymphoid cells per anlage (based on four cultures) was estimated on days 0 2 4 6 8 10 and 12 of culture

Two representative growth curves are illustrated in Fig 2 An apparently exponential increase in the number of lymphoid cells per anlage was noted between days 0–6 The doubling time was approximately 21 hours

After day 6, the exponential increase ceased and the number of lymphocytes per anlage reached a plateau value which lasted from day 6–8 until at least day 12 of culture

Influence of medium composition and subcultivation In a previous work (1) Waymouth's MB 752/1 tissue culture medium was used for the organ cultures Table 1 shows that this medium and the more complicated RPMI 1640 medium used in the present investigation equally well supported the development of a lymphoid thymus at least between days 2 and 8 of culture

In order to investigate whether the plateau value of the number of lymphocytes, demonstrated in Fig 2, possibly depended on unfavourable growth conditions due to medium depletion or the increase in size of the anlagen in culture, the following type of experiment was performed

Organ cultures of the thymic anlagen of

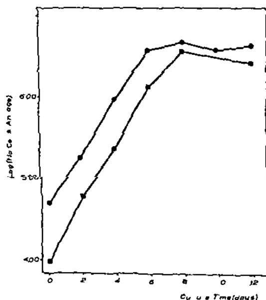


Fig 2 Time dependent lymphoid development (Log [No cells/anlage]) of thymic anlagen of 10-day-old chick embryos in organ culture (two experiments) Each point represents the mean of four cultures

TABLE 1 *Lymphoid Development (Log[No Cells/Anlage]) of Thymic Anlagen of 10 Day Old Chick Embryos in Organ Culture Using Waymouth's MB 752/1 or RPMI 1640 Medium*

Medium	Log (number of cells per anlage)* day 2	day 4	day 6	day 8
Waymouth's MB 752/1	5 23	5 77	6 26	6 51
RPMI	5 00	5 72	6 25	6 51

* Mean of four pooled anlagen

10 day-old chick embryos were initiated On day 6 of culture, ⅔ of the thymic anlagen were dissected free of Spongostan and half of these were transferred to fresh organ culture dishes Each anlage of the other half was divided into 5 fragments of equal size and each fragment was transferred to a new organ culture dish As controls one third of the organ cultures of thymic anlagen received a medium change only but were otherwise left intact A complete change of medium was done on day 8 of culture The number of lymphoid cells per whole anlage and the value for the combined fragments of an anlage was determined on days 6 8 and 10 of culture Two experiments were performed

The results are given in Table 2 In all three groups transferred whole or divided as well as non transferred control anlagen the mean number of cells per thymic anlage in

TABLE 2 *Effect of Subcultivation of Whole and Divided Organ Cultured Thymic Anlagen of 10 Day Old Chick Embryos**

Culture time (days)	Transferred anlagen whole§	divided§	Non transferred whole anlagen§
6	—	—	6 47±0 06
8	6 70±0 14	6 64±0 14	6 70±0 06
10	6 48+0 24	6 39±0 37	6 77±0 13

* Subcultivation (transfer) of whole or divided anlagen on day 6 Medium changes on day 6 and 8

§ Mean (log [no cells/anlage]) ± S D of four anlagen

creased significantly between culture days 6 and 8 ($P < 0\,02$ $< 0\,05$ and $< 0\,001$, respectively) No significant increase occurred between days 8 and 10 Although transferred anlagen on day 10 appeared to contain somewhat smaller numbers of lymphoid cells no statistically significant difference between the three groups was noted at either day 8 or day 10

Thus subcultivation of whole or divided thymic anlagen and medium changes did not significantly increase the number of lymphoid cells per anlage

Influence of serum concentration The effect of the concentration of whole serum on the development of thymic lymphocytes in organ culture was investigated The thymic anlagen of 10 day old chick embryos were cultured for 6 days with different serum concentrations in the medium (0 625 to 20 per cent in two fold dilutions) and the number of lymphocytes per anlage was determined Five separate experiments were performed

The results of a typical experiment are demonstrated in Fig 3 The number of lymphocytes per anlage increased with increasing concentration of serum The thymic anlagen of serum free organ cultures contained insignificant numbers of lymphoid cells ($< 10^4$) Already at the lowest concentration tested (0 625 per cent) a significant increase of lymphocytes above control levels was noted

The shape of the dose response curve appeared biphasic if the mean number of lymphoid cells per anlage was plotted against the logarithm of the serum concentration A less steep slope between 0 625 per cent and 2 5 per cent serum was generally noted In 3 of the 5 experiments a peak was noted at 1 25 per cent serum Between 2 5 per cent and 20 per cent the slope was steeper In the analysis of 11 dose response curves (not included in this report) within this concentration range it approached linearity The linearity was more complete in the log log plot (not shown) of cell number versus serum concentration

Delay of serum addition Preliminary in

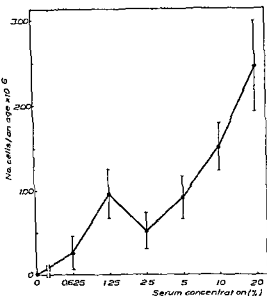


Fig 3 Effect of the serum concentration on the lymphoid cell yield (No cells/anlage × 10 ⁶) of thymic anlagen of 10 day-old chick embryos after 6 days in organ culture Each point represents the mean ± S D of four cultures

vestigations to define the role of serum in the development of the lymphoid thymus *in vitro* were carried out

For this purpose, thymic organ cultures were initiated in the usual way but with serum-free medium Chick serum (10 per cent) was added either on day 0, 1 or 2 of culture and the number of lymphoid cells per anlage was assayed either 3, 4 or 5 days after serum addition Two experiments were performed

The results are summarized in Fig 4 The delayed addition of serum, either on day 1 or 2 of culture, resulted in a corresponding delay of the exponential increase of the number of lymphoid cells per anlage The time dependent rate of increase in the number of lymphocytes per anlage was not changed by the delay of serum addition Furthermore, the same or a slightly lower number of lymphocytes per anlage was attained at the same time after serum addition, whether the latter was done on day 0 or delayed by 1 or 2 days

Removal of serum In order to test the importance of the continued presence of serum for the lymphoid development of the thymus *in vitro* the following type of experiment was performed

Organ cultures of 10 day-old embryonic thymic anlagen with 10 per cent chicken serum were initiated On days 3, 4 and 5, the cultured anlagen were dissected free of surrounding Spongostan, washed once in serum-free RPMI 1640 medium and transferred to new complete organ culture assemblies, this time without serum Control anlagen were transferred to serum containing cultures Cell counts on 4 separate non transferred anlagen in serum containing medium were performed after harvest on days 3 4, 5 and 6 The cultures with transferred anlagen were all harvested on day 6 and the number of lymphoid cells per anlage was determined Four separate anlagen were used for each treatment Three experiments were performed

The results are demonstrated in Fig 5 The results of the separate experiments did not differ significantly and they were therefore pooled After removal of serum, the number of lymphoid cells per anlage re-

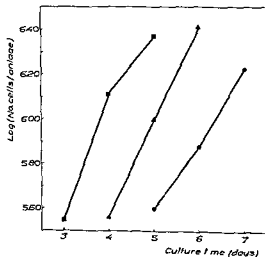


Fig 4 Effect of delay of addition of serum on the time dependent lymphoid development (Log [No cells/anlage]) of the thymic anlagen of 10 day-old chick embryos in organ cultures Serum added on culture day 0 (■), 1 (▲) and 2 (●) Each point is the mean of four cultures. The no of lymphoid cells was determined 3 4 and 5 days after serum addition

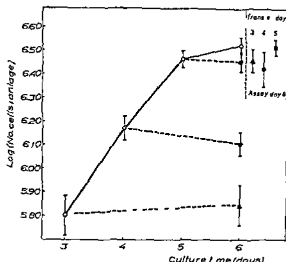


Fig 5 Effect of transfer to serum free cultures on the time dependent lymphoid development (mean log [No cells/anlage] ± S D) of the thymic anlagen of 10 day old chick embryos in organ culture Nontransferred control anlagen (○), Anlagen transferred to serum free cultures on day 3 (▲), day 4 (●) and day 5 (■), Anlagen transferred to serum containing cultures on days 3 4 and 5 in upper right corner (assayed on day 6)

mained at the value recorded at the time of transfer to serum free medium and no statistically significant increase or decrease was noted The procedure of transfer to serum containing cultures had possibly an inhibitory effect on the subsequent development of the thymus, although this was not statistically significant

If thymic anlagen transferred to serum free and serum containing organ cultures were compared the former were seen to contain a significantly smaller number of lymphoid cells on day 6 of culture when transferred on day 3 ($t = 5.81$, d f $= 22$, $P < 0.001$) and day 4 ($t = 3.77$, d f $= 18$, $P < 0.005$) but not on day 5 ($t = 1.26$, d f $= 18$, $P > 0.05$)

Thus, removal of serum from the organ cultures arrested the normally occurring increase of lymphoid cells per thymic anlage

DISCUSSION

The present investigation demonstrates that the organ culture technique previously described (1) for the *in vitro* development of the embryonic chicken thymus gives a high yield of lymphoid cells, approximately 3.50×10^6 cells per anlage after 6 days in culture

The reproducibility within and between experiments was of a degree allowing that the number of lymphoid cells per thymic anlage at different times of culture, determined by counting the cells in suspensions of homogenized anlagen, could be used as a reasonably accurate quantitative measure of thymic development

The time dependent increase in the number of lymphoid cells per thymic anlage was characterized by two distinct phases The initial exponential phase most likely started at the time of initiation of the cultures and lasted approximately for 6 days The doubling time for the lymphoid cell population was approximately 21 hours The generation time of the lymphoid cells must, however, be defined in order to draw any accurate conclusions as to the cell kinetics in the thymic anlagen

The exponential growth phase was followed by a plateau phase commencing between days 6 and 8, and lasting until at least day 12 of culture During this interval, no further increase in the number of lymphoid cells per thymic anlage was demonstrated The main reason for this may be a decreased cell proliferation (1) It cannot be excluded that a loss of cells, either by increased rate of cell death or escape of lymphocytes from the anlagen may contribute

We can offer the following explanations for the termination of the exponential and beginning of the plateau phase during thymic development *in vitro* Firstly for technical reasons the organ culture system may not support proliferation of lymphoid cells beyond days 6–8 of culture Thus the gradual increase in size and metabolic activity of the anlagen may result in a poor exchange of nutrients and metabolic waste products between the anlagen and the tissue culture medium or a depletion of medium nutrients

Secondly, the supply of lymphoid precursor cells present in the thymic anlagen at the ini

tiation of the organ cultures may be exhausted in 6 days (or less)

Thirdly, the micro environment provided by the epithelial thymic anlage may only allow a certain number of lymphoid cells When this is reached, cell proliferation ceases, e g due to a feedback mechanism

The finding that subcultivation of whole or divided thymic anlagen and medium changes did not increase the total number of lymphoid cells per anlage above the plateau value argues against the first explanation Furthermore, after day 6 of culture, most lymphoid cells differentiate into small lymphocytes (1) which by days 10–12 of culture constitute the majority of the thymic lymphoid cells This fact and the finding that these cells form 'caps' with fluorescein labelled concanavalin A (19) and react to lymphocyte mitogens *in vitro* (19, 20) *indicate that the culture* conditions at least are sufficient for differentiation and maintenance of the small lymphocytes Still, more detailed cell kinetic studies of cell proliferation and cell death during the plateau phase are warranted

The second explanation, i e depletion of the lymphoid precursor cell pool is quite plausible and was suggested by *Mandel* & *Russell* (12) to account for the poor long time survival of embryonic mouse thymus in organ culture It could possibly directly be tested by supplying the cultured thymic anlagen on days 6–8 with fresh precursor cells which would then increase the number of lymphoid cells above the plateau level

The third explanation i e that the thymic micro environment accepts only a certain number of lymphoid cells may be of physiological significance *Metcalf* (13, 14) has reviewed existing evidence according to which the regulation of thymic lymphopoiesis normally is autonomous i e at least mainly controlled by the thymus itself *In vivo*, either during the embryonic and perinatal period (2) or during thymic regeneration after involution caused by total body irradiation (10) the thymus shows an exponential increase in the number of lymphoid cells with extensive cell proliferation followed by a plateau with steady state lymphopoiesis and development of small lymphocytes This may correspond to our findings obtained during the growth of the embryonic thymus in organ culture If the number of lymphocytes by a feed back mechanism controls the rate of lymphopoiesis, one would expect an experimental depletion of lymphocytes to be followed by their regeneration in the organ cultured thymus during the plateau phase

In a previous investigation (1) we found that the embryonic chicken thymus required only chicken serum as medium supplement for the lymphoid development to take place in organ culture The present investigation demonstrated that the lymphoid development *in vitro* completely depended on the presence of chicken serum An increase of the serum concentration in the organ culture medium *resulted in an increased number of lymphoid* cells per thymic anlage At least within a concentration range of 2 5 to 20 per cent serum in the culture medium this relation appeared linear in a log log plot This suggests that the organ cultures may be used as a quantitative assay for the thymus stimulating activity of serum or serum preparations

Embryonic thymic anlagen in serum free organ cultures did not display any significant development of lymphoid cells, but were still able to develop into lymphoid structures if chicken serum was added on day 1 or 2 of culture This suggests that serum is not necessary to maintain the lymphoid precursor cells *in vitro* Using a hot pulse technique with H^3 methyl thymidine we have shown (*Juhlin* & *Alm*, Cell Tissue Kinet, in press) that lymphoid precursor cells which are in cell cycle in the thymic anlage of 10 day-embryos remain in cell cycle during serum free culture conditions This suggests that chicken serum initiates the lymphoid development of the embryonic thymus in organ culture

The effects of the removal of serum from the organ cultures, e g a cessation of the lymphoid development of the thymus, indicates further that serum is necessary also

for the maintenance of thymic lymphopoiesis *in vitro*

The mode of action of the thymus stimulating activity in serum (TSAS) remains to be determined It may be the effect of one or several factors vital to the general survival, or differentiation of cells *in vitro* It may also be required specifically for the lymphoid development of the thymus in organ culture It is thus possible that the TSAS is due to a lymphopoietin, akin to erythropoietin and the colony stimulating factor (14) The organ culture technique used in the present investigation should facilitate the further characterization of the thymus stimulating activity in serum, its mode of action and possible physiological importance In a following communication some of the properties of the TSAS are described (*Sällström*, to be published)

It appears that thymic lymphopoiesis, in particular its regulation and the morphological and functional development of lymphocytes, with advantage can be studied *in vitro* using the organ culture technique

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We wish to thank Miss *Marianne Eriksson*, Miss *Lili Anne Eriksson* and Mrs *Eva Beckman* for excellent technical assistance

This work was supported by grants from the *Swedish Cancer Society* (proj no 511 B72 O2XA), the *Expressens prenatalfond* and the *Faculty of Mathematics and Sciences of the University of Uppsala*

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DIFFUSE PLEURAL MESOTHELIOMA WITH DISTANT BONE METASTASIS

A Case Report

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A diffuse malignant mixed type of pleural mesothelioma with distant bone metastasis is reported Distant bone metastasis has only been recorded once in the literature but not adequately documented Metastases in this patient were with one exception all osseous Staining properties of the pleural tumour and metastases are compared All metastases showed epithelial like and fibrosarcomatous component with staining properties similar to the pleural tumour

Diffuse malignant mesothelioma is a relatively rare tumour which does not usually become widely disseminated *Hourihane* (1964) stated that all bone deposits in his series occurred by continuity The case here reported shows an unusual degree of bone involvement with a distant metastasis to the right humerus

MATERIAL AND METHODS

A 56 year old Caucasian man was admitted with increasing breathlessness and a dull continuous ache over the right posterior chest during the preceding 6 months He had worked all his life as a heating engineer, and smoked 20 cigarettes/day up to 7 years previously In May 1972 there was a sudden onset of severe right pleuritic pain that cleared spontaneously in 3–4 days In July 1972 a dull ache developed over the right side of the chest Chest x ray showed an apparent pleural effusion but an attempt to aspirate fluid was unsuccessful Radiographs showed right pleural thickening with erosion of the 5th rib, loculated effusion, and a sclerotic lesion in both the metaphysis of the right humerus and in T_{12}

A complete necropsy was done on a normally developed well nourished Caucasian The right pleural cavity was completely obliterated by firm white tumour tissue with maximal involvement over the lower lobe and extending into the interlobar fissures There was only superficial invasion and moderate compression of the lung parenchyma Tumour infiltrated by continuity into the right 5th rib right parietal aspect of the pericardium and diaphragm extending to the right of the aorta into the abdominal cavity to the level of D_1. There was a loculated serous pleural effusion between the right lower lobe and the diaphragm The left parietal pleura showed extensive coalescent asbestos plaques and the bronchial tree contained a moderate amount of mucopus The vertebral bodies from C_7 to L_5 showed partial or complete replacement by firm white tumour that also replaced most of the head of the right humerus (Fig 1) There was no continuity between the pleural tumour and the humeral lesion The only other metastasis was a white nodule 2 cm diameter in the right lobe of the liver For histological study multiple sections were stained with haematoxylin and eosin PAS, Alcian Blue Colloidal Iron, Masson trichrome, Weigert stain for elastic fibres and reticulin stain

The presence of asbestos bodies was confirmed by the use of smears, unstained 30 µ sections and a new method to be reported separately

Received 25 x 73 Accepted 24 xi 73

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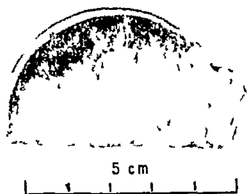


Fig 1 Section of the humeral head showing replacement by tumour



Fig 2 Epithelial like and stromal cells with similar nuclei containing prominent nucleoli Haematoxylin and eosin × 535

RESULTS

The pleural tumour was a diffuse malignant mesothelioma mixed type The epithelial like component consisted mainly of branching tubules surrounded by dense or loose sarcomatous stroma (Fig 2) Special stains showed increased reticulin and collagen but no elastic tissue excess The epithelial like component was PAS negative and stained with Alcian Blue and Colloidal Iron In addition Alcian Blue and Colloidal Iron stains showed focal areas of positivity both in lacunae and in nests of epithelial like cells embedded in the connective stroma All bone metastases showed epithelial like and fibromatous component with similar staining properties to the pleural tumour There was no difference between the distant metastasis and those that developed by continuity The histology of the liver metastasis was identical with the osseous one

DISCUSSION

A review of the literature dealing mainly with the study of large series of cases (*Poulsen & Sorensen* 1959 *Winslow & Taylor* 1960 *Butt* 1962 *Semb* 1963 *Manguikian & Prior* 1963 *Hourihane* 1964 *Urscheel & Paulsen* 1965 and *Schlienger et al* 1969) revealed only a solitary case with distant bone metastasis Case No 33 of *Schlienger et al* (1969) had a metastasis in the tibia the histology of which was not reported This is the first case with distant bone metastases to have histological confirmation *Hourihane* (1964) stated that bone lesions by continuity were fibrosarcomatous in type and that metastases to lymph nodes were mostly of epithelial type regardless of the histology of the primary tumour The present case shows that the epithelial like component can be present in both types of bone metastases

Winslow & Taylor (1960) following *Meyer & Chaffee* (1939) stated that hyaluronic acid could be demonstrated in stromal connective tissue of mesotheliomas Later *Wagner et al* (1962) regarding this as non specific considered the presence of hyaluronic acid with in cells or cystic spaces a more specific feature The presence of epithelial like cells in both types of bone metastases was con

firmed by the use of the above mentioned findings

CONCLUSION

A case of malignant mesothelioma with histologically confirmed distant bone metastasis is reported The presence of epithelial-like cells and acid mucopolysaccharides in both types of bone metastases was established

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This work was carried out with technical help from Miss *Sidsel Heieren* on grant from the *Norwegian Cancer Society*

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Acta path microbiol scand Section A 82, 299-310, 1974

ULTRASTRUCTURAL STUDY OF HETEROKARYONS FROM ROUS RAT SARCOMA CELLS AND NORMAL CHICKEN CELLS

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Rous virus transformed rat cells containing the Rous virus genome but lacking the ability to produce Rous virus particles have been fused with chicken erythrocytes chicken lymphocytes or chicken fibroblasts using Sendai virus Heterokaryotic cells with both rat sarcoma cell nuclei and chicken cell nuclei have been studied in the electron microscope Shortly after the fusion a series of changes in the chicken nuclei are demonstrable, which as seen in the electron microscope imply a 'reactivation" and production of intranuclear RNA containing structures as well as a development of nucleoli In parallel, the chicken nucleus increases in size and the chromatin is transformed from condensed aggregates into a finely dispersed material In spite of all evidence of a protein synthesis in the heterokaryotic Rous sarcoma cell directed from the chicken nucleus there is no formation of virus particles in cells with Rous rat sarcoma cells and chicken erythrocytes or chicken lymphocytes In cultures with Rous rat sarcoma cell—chicken fibroblast heterokaryons a regular synthesis was seen It seems apparent that the chicken fibroblast contains some factor, whether genetically coded or not that is required if a production of complete Rous virus particles is to be achieved

The full expression of the viral genome which is integrated in Rous virus transformed mammalian sarcoma cells can be achieved in different ways Back transfer to chickens of mammalian tumour tissue is known to elicit virus producing chicken sarcomas (*Ahlström* 1964, *Zilber* 1965) as well as co cultivation *in vitro* between mammalian sarcoma cells and suitable chicken cells as target cells (*Simkovic et al* 1962, *Svoboda et al* 1963, *Vigier* & *Svoboda* 1965) Inoculation of mammalian sarcoma cells on the chorio-allantoic membrane in chickens produces a chicken sarcoma cell with capacity of producing infectious virus after down growth and contact has been established between the mammalian cell and a mesodermal chicken cell (*Vrba* & *Chaytor* 1964, *Lindberg* 1970)

A close contact between mammalian sarcoma cells and chicken cells which progresses to cell fusion and formation of heterokaryons can be obtained by inactivated Sendai virus This is known to increase the yield of infectious Rous virus, but so far the virus production has been obtained only if the normal cell has been a chicken fibroblast (*Svoboda et al* 1967, *Vigier* 1967, *Machala et al* 1970) It is not clearly established whether the increased virus synthesis is due to a synthesis augmented by the agglutinating capacity of Sendai virus, which presumably

Received 14 viii 73 Accepted 27 xi 73

might increase the contact between the mammalian sarcoma cell and suitable chicken cells, or the increased virus production results from a synthesis restricted to the newly formed heterokaryotic cells In the present study, mammalian sarcoma cells containing the Rous virus genome are fused with normal chicken cells in order to obtain Rous virus particles In addition, an ultrastructural analysis of heterokaryotic cells made from Rous rat sarcoma cells and embryonal or adult erythrocytes, lymphocytes or normal chicken fibroblasts, respectively, is made at various intervals to see whether there are morphologic changes that can be related to the production of virus particles Cell cultures of heterokaryotic cells were established *in vitro* and, at regular intervals, samples were processed for electron microscopy and biological tests

MATERIAL AND METHODS

RR cells A RSV SR induced rat sarcoma propagated at the institute for several years was explanted and adjusted to *in vitro* cultures Its capacity to elicit virus producing chicken sarcomas was tested at regular intervals through backtransfers to chickens

Chicken erythrocytes lymphocytes and fibroblasts Embryonal erythrocytes were obtained from 14 16 days old embryos through direct heart puncture Adult erythrocytes were withdrawn by venous puncture Lymphocytes were prepared from the Fabrician bursa of 1–3 days old chickens

Rous virus sensitive chicken fibroblasts were cultured from 9–11 days old chicken embryos

Fusion of RR cells with chicken cells RR sarcoma cell suspensions were irradiated with 3 000 rad to prevent overgrowth of the non fused RR cells Suspensions of irradiated cells in saline with 1×10^7 cells per ml were mixed with equal volumes of erythrocyte lymphocyte or chicken fibroblast suspensions in saline containing 3×10^7 cells per ml The mixture was cooled to 0° C and UV inactivated Sendai virus with a haemagglutination titre of 1 2 500 was added in the same volume of saline thoroughly mixed and transferred to a water bath at +37° C After 15 minutes of continuous shaking the suspensions were washed in saline and transferred to plastic dishes for further culturing

Cell cultures Cell cultures were established in plastic dishes of 60 mm in diameter (Falcon) As medium was used Eagle's MEM with 9 6 gram sodium bicarbonate added per litre and 10 per cent calf serum inactivated at +56° C for 30 minutes as extra requirements Every third or fourth day the medium was exchanged All cultures were grown in an atmosphere containing 5 per cent CO

Biological tests Six weeks old chickens were inoculated with culture medium withdrawn from the plastic dishes every second day in an amount of 0 5 ml undiluted medium in each pectoral muscle All chickens were taken from a RIF-free stock If no signs of tumour growth were noted at the injection site the animals were observed for at least three months

Electron microscopy The day after the fusion of the cells, samples were taken from the cultures for ultrastructural processing, samples were then prepared every second or third day until the viability of the cells declined All specimens were fixed in veronal buffered glutaraldehyde Rheomacrodex solution over night at +4° C (veronal buffered saline according to *Sjostrand* (1967), 1 5 per cent glutaraldehyde and 4 per cent Rheomacrodex (Pharmacia)), dehydrated in acetone and embedded in Vestopal W according to *Ryter* & *Kellenberger* (1958) Sectioning was performed on a LKB Ultrotome III and all specimens were post stained in uranyl acetate for 45 minutes at +60° C (saturated solution in Aq dest) and in lead citrate according to *Venable* & *Coggeshall* (1965) The ultrastructural examination was made in a Zeiss EM 9 A

RESULTS

In all experiments, heterokaryons were regularly obtained In general there were more than one nucleus of each type in each heterokaryon—one or several RR-cell nuclei and two or more red cell nuclei Lymphocyte nuclei were only occasionally multiple but in rare instances up to five lymphocyte nuclei were encountered in the same heterokaryon In addition multinucleated cells were fre

Fig 1 A Rous sarcoma cell with a chicken erythrocyte nucleus (right) and part of the sarcoma cell nucleus (left) One day after fusion × 28 000

Fig 2 RR cell with two sarcoma cell nuclei (top and lower left) and one erythrocyte nucleus three days after the fusion Centrally a lighter area with an aggregate of interchromatin granules are seen and at the border zone larger perichromatin granules are found At the periphery the chromatin is partly dispersed into a finely granular material × 20 000


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quently observed—cells with two or more nuclei of the same type There are no difficulties in recognizing the difference between the RR cell nucleus and the chicken nucleus whether it is from erythrocytes lymphocytes or chicken fibroblasts During the first 2 3 days after the fusion there are clear differences in size, staining characteristics and shape of the two nuclei The chicken nucleus, either red cell nucleus or lymphocyte nucleus is always rounded with a smooth, regular outline close to a spheroid shape without infoldings of the nuclear membrane The nuclear chromatin is dense and is heavily stained (Fig 1) Occasionally a homogeneous dark nucleus is observed Both in red cell nuclei and in lymphocyte nuclei a loose central area develops (Fig 2 3) This is seen about three days after the fusion in the erythrocyte nucleus and in the lymphocyte nucleus about 5–7 days after the fusion In all nuclei so far observed, there are aggregates of a granular material in this lightly stained area during the initial phase of the nuclear enlargement The granules have the size of interchromatin granules (about 200–250 Å) and their appearance conform to previous description of this nuclear component (*Monneron & Bernhard* 1969) At the periphery of the loose central area adjacent to the finely dispersed chromatin there are coarser granules occasionally forming irregular aggregates along the border zone (Fig 2, 4) These granules have diameters varying between 400 500 Å and their appearance and location conform well to the appearance of perichromatin granules (*Monneron & Bernhard* 1969) With time the chicken nuclei enlarge and the condensed chromatin dissolves into areas of finely dispersed DNA Aggregates of interchromatin granules still remain in interchromatin areas (Fig 4) When nucleoli develop these aggregates may remain but are in general less prominent, and eventually they disappear as aggregates Interchromatin granules, however, can be found in all nuclei The development of nucleoli is seen to occur very close to the aggregates of interchromatin granules but in general a small intermediate zone remains in between (Fig 4–5) In the 'early' heterokaryons almost every chicken nucleus is spherical in shape, which is in clear contrast to the irregular shape of most RR-cell nuclei (Fig 2, 6) Nuclear pores are regularly seen but in some nuclei they might be difficult to observe After a culture period of 4–6 days after the fusion, the red cell nuclei of chicken origin behave in two different ways Either they remain small darkly stained and with time they shrink and become dissolved or the nuclei enlarge considerably, presumably by an inflow of water and proteins The chromatin of these nuclei is gradually dispersed and finally almost no clumping or margination of chromatin along the nuclear membrane is observed In almost every nucleus there is a nucleolus after a period of 7–10 days after the fusion A future development of nucleoli can be anticipated as early as 3 days after the fusion but full size and functional appearance is not achieved before 10 12 days after the fusion (Fig 6–7) Multiple nucleoli are not found in any of the chicken nuclei so far observed The chicken nuclei remain rounded in many cells but the nuclear membrane is frequently curved and minor infoldings give the nuclear membrane a wavy outline (Fig 7) Deep and angular infoldings of the type which is found in the RR cell nuclei are not seen in the chicken nuclei

After 7–13 days of cultivation, most 'inactive' chicken nuclei which have been in

Fig 3 A chicken lymphocyte nucleus seven days after the fusion The size of the interchromatin granules is approximately 200–250 Å and of perichromatin granules around 400–500 Å in diameter The perichromatin granules are in this nucleus located in the interchromatin area as well and they are associated with fine fibrillar structures presumably interchromatin fibrils × 61 000

Fig 4 A chicken erythrocyte nucleus in a heterokaryon three days after the fusion The interchromatin areas are partly formed and the developing nucleolus consists mostly of an amorphous material × 29 000

corporated into RR cells display considerable degenerative changes such as shrinking of the nuclear membrane, fragmentation and partial disintegration of the chromatin Later bodies resembling autophagosomes and containing nuclear debris are found Occasionally a completely round nucleus can be found which has a smooth outer membrane and a chromatin structure that cannot be differentiated from the chromatin pattern that is typical for RR cell nuclei (Fig 8) Only the shape suggests that it is a nucleus of chicken origin In addition it is impossible at this stage to differentiate between lymphocyte and erythrocyte nuclei as the characteristic appearance of these nuclei is no longer discernible after the increase in volume and loosening of the chromatin texture of the nucleus

In summary the RR cell heterokaryons with chicken erythrocytes or with chicken lymphocytes undergo a series of transformation steps as regards the incorporated nucleus The small chromatin dense, spheroid chicken nucleus enlarges and aquires an irregular outline with infoldings and irregularities of the nuclear membranes The nuclear chromatin dissolves from condensed clumps into a finely dispersed pattern and in the centre of the nucleus a prominent nucleolus is formed This development involves in general a short intermediate step during which aggregates of interchromatin granules appear closely associated with the nucleolar area but later disappear as aggregates when the nucleolus is fully developed Under the present conditions with pre irradiated RR cells this development is completed in most cells after 12–15 days after the fusion In the cytoplasm of these heterokaryons no particular findings are observed As the RR cells are irradiated there are numerous vesicles in the cytoplasm and in addition myelin like structures are frequently observed No traces of virus formation have been encountered either at the plasma membrane or in intracytoplasmic vesicles

In all biological tests where RR cells were injected into chickens virus producing sarcomas were obtained All samples of culture medium proved to be entirely free of infectious Rous virus if they were drawn from cultures of heterokaryotic cells made from RR sarcoma cells and chicken erythrocytes or chicken lymphocytes

Contrary to the above findings a regular production of infective Rous virus particles could be demonstrated in tissue cultures with heterokaryons made from RR sarcoma cells and chicken embryo fibroblasts The earliest biological confirmation was seen on the fourth day after the fusion—cell free medium from the tissue cultures elicited virus producing chicken sarcomas if inoculated into test chickens The ultrastructural changes in heterokaryons of chicken fibroblasts and RR sarcoma cells start with an activation of the chicken nucleus in much the same way as described above for chicken erythrocyte or chicken lymphocyte nucleus Thus there is a loosening of the condensed nuclear chromatin and this is followed by a formation of perichromatin and interchromatin granules and a prominent nucleolus (Fig 9–10)

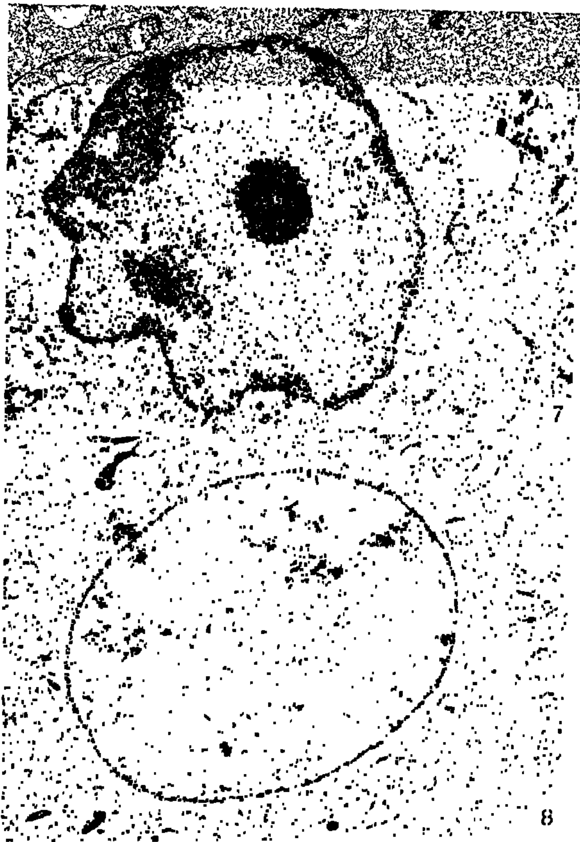
If virus production is found in biological tests there are pronounced degenerative changes in most cells in the culture dishes There are numerous and large vacuoles in the cytoplasm of both heterokaryotic and other cells and fragmentation of cell membranes disintegration of mitochondria and separation of the membranes of the endoplasmic reticulum is frequently observed

No conspicuous changes are found in the cytoplasm of RR sarcoma chicken fibroblast heterokaryons that can be related to a synthesis of virus particles RSV particles are

Fig 5 A chicken nucleus with both interchromatin granules and a nucleolus under development × 63 000

Fig 6 A RR-cell with two highly irregular nucleolated sarcoma nuclei and one erythrocyte nucleus 13 days after fusion The nucleolus of the chicken nucleus (right) is prominent but not of the giant size as the nucleolus of the partner RR-cell nucleus × 7 900


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not observed in vacuoles in the cytoplasm of heterokaryons and no virus particles are found at the plasma membranes of these cells However, a few virus particles the size and shape of which are compatible with Rous virus particles are seen close to fragments of dead cells The morphology of these fragments is unfortunately so badly destroyed that it is impossible to decide whether they derive from heterokaryotic cells or non fused chicken fibroblasts

Well preserved heterokaryotic rat sarcoma—chicken fibroblasts could not be found later than seven days after the fusion and in most experiments the cell cultures were impossible to maintain for periods longer than 9 10 days after the fusion

DISCUSSION

The purpose of the present investigation was to obtain Rous virus production from hetero karyotic cells with a mammalian Rous sar coma cell containing the Rous virus genome and a normal chicken nucleus It has been found previously that infectious Rous virus could not be demonstrated in biological tests when red cell nuclei from chicken were in corporated into Rous Chinese hamster sar coma cells (*Machala et al* 1970, *Svoboda et al* 1971) The present results confirm these observations and in addition it is found that chicken lymphocyte nuclei lack the abil ity to direct a synthesis of complete Rous virus particles There was no trace of forma tion of viruses belonging to the avian leucosis group, and an abortive synthesis did not ap pear to take place, neither was there a pro duction of viruses that can be suspected to belong to the helper group of viruses often encountered together with Rous virus Avail able data (*Sarma et al* 1966, *Vigier & Montagnier* 1966 *Vigier* 1967) indicate that mammalian sarcoma cell lacks the ability to synthesize the viral envelope but not the viral homologous DNA, which seems to be distributed to all daughter cells after a mitotic division

Neither the chicken red cell nucleus nor the chicken lymphocyte nucleus can contrib ute all those factors to the heterokaryotic cell that are necessary for a synthesis of a complete Rous virus particle—even if the chicken nucleus is fully "activated" with a development of prominent nucleoli indicating an active metabolism directed from a previously dormant and inactive chicken nucleus How ever, the heterokaryotic cell composed of RR-sarcoma cells and chicken fibroblasts contains both cytoplasmic and nuclear components from both parent cells This implies that the lacking component might be furnished by the cytoplasm of the fibroblast or as suggested by some observers (*Sarma et al* 1966, *Vigier* 1967), by some components of the plasma membrane of the chicken fibroblast

In the present study it has not been possi ble to establish that heterokaryotic RR-sar coma chicken fibroblasts *do* produce RSV particles As found in previous studies, however, it seems quite obvious that the hetero-karyotic *cells produce viral antigens as de* monstrated with fluorescent antibody tech nique (*Machala et al* 1970) and it is im possible from the present observations to exclude a production of RSV from the hetero karyotic cells as the number of virusproducing heterokaryotic cells might be low It can be stated, however, that up till that moment when degenerative cell alterations no longer permit a morphological analysis in virusproducing cultures there are no obvious cell alterations in most heterokaryotic cells which

Fig 7 *A RR-cell with an incorporated erythrocyte* nucleus 17 days after the fusion The nuclear membrane is infolded and slightly irregular The chromatin is partly condensed and both inter chromatin and perichromatin granules can be dis cerned × 24 000

Fig 8 An almost spheroid nucleus presumably of chicken origin in a RR cell 17 days after the fusion between RR cells and chicken lymphocytes The chromatin is finely dispersed and no margination of the chromatin along the nuclear membrane is seen. A few small chromatin aggregates remains often associated with perichromatin granules × 20 000

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distinguish them from heterokaryotic cells in non virus producing cell cultures

The series of nuclear "activation" steps as seen in the present study are in accordance with the original light microscopic observations by *Harris* (1968) and *Harris et al* (1969) of the transformation of erythrocyte nuclei from chicken after incorporation into the cytoplasm of active cells Under the present conditions the partner cell is a malignant sarcoma cell of mammalian origin, but even then the general pathway of activation is retained as it originally was described The regular appearance of a granular intranuclear material (both interchromatin and perichromatin granules) closely associated with the initial steps in the formation of a nucleolus is noteworthy As interpreted previously (*Monneron & Bernhard* 1969) these granules represent intranuclear RNA and from a morphological viewpoint it is evident that the 'activated' chicken nucleus is capable of and presumably is directing a metabolism of its own This is in agreement with the previous demonstration of chicken antigens in heterokaryons (*Harris* 1968, *Harris et al* 1969) and by the fluorescent antibody techniques (*Machala et al* 1970) in the Rous Chinese hamster sarcoma—chicken erythrocyte heterokaryon of presumed Rous virus antigen This further confirms that the "activated' chicken nucleus participates in the protein synthesis in the cytoplasm of the foreign host cell

In some experiments the malignant character seems to diminish after fusion of a malignant cell with a non malignant cell (*Bregula et al* 1971, *Klein et al* 1971, *Wiener et al* 1971) As suggested by the irregular appearance of the chicken nucleus after 8–10 days after the fusion and by the highly irregular RR cell nuclei in most heterokaryons the malignant character might remain unchanged It might be argued that the hetero karyotic cell must divide one or several times before any apparent change in its behaviour as regards malignancy can be noted

An ultrastructural analysis of a rapid dynamic process gives only very few glimpses of a series of events that are under continuous changes A further difficulty often encountered is the unability to confirm low frequent observations with biological tests Thus it seems likely that an ultrastructural analysis of heterokaryotic cells and the dramatic changes that are observed in these cells after the fusion of Rous rat sarcoma cells with normal chicken cells must be complemented by other methods allowing a biological interpretation of the results

Fig 9 A chicken fibroblast fused with a Rous rat sarcoma cell The chicken fibroblast nucleus (right) has acquired a small nucleolus 5 days after the fusion and most of its chromatin is dispersed No RSV particles were found in this specimen although the cell free culture medium contained infectious RSV × 7,500

Fig 10 A chicken fibroblast—Rous rat sarcoma cell heterokaryon 5 days after the fusion Two chicken cell nuclei are seen in the late phase of nuclear 'activation A small part of the much larger nucleus of rat sarcoma origin is seen (upper right corner) × 13 300

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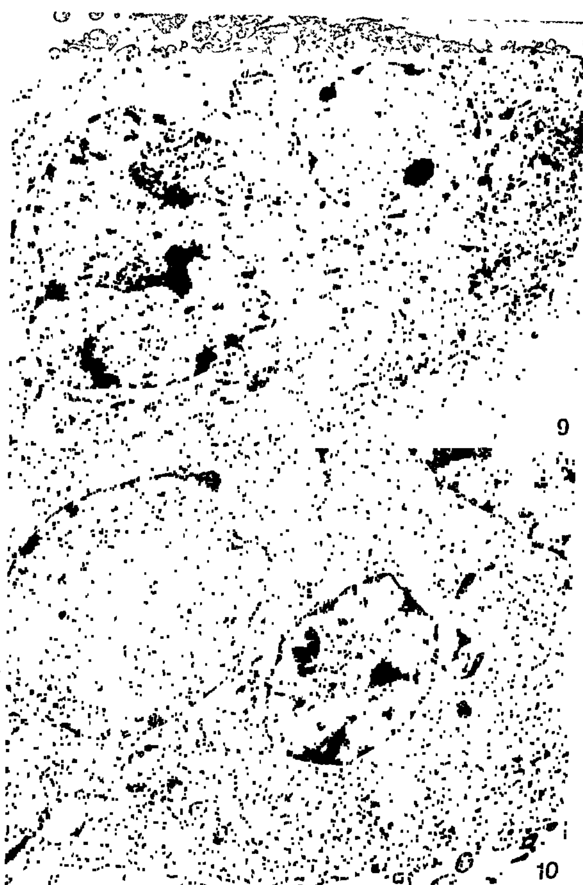
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Acta path microbiol scand Section A 82, 311 318, 1974

LEAD INDUCED INJURY OF *IN VITRO* CULTURED RAT FIBROBLASTS

A Light and Electron Microscopical Study

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In vitro cultured embryonal rat fibroblasts were used for the testing of a concentration of lead ions which would cause widespread cellular injury but not disseminated cell death Of the tested dosages between 1 and 100 μg Pb (NO_3) /ml cultivation medium, 25 μg was deemed suitable for further studies of the mechanism behind the cellular injury of lead Electron microscopically there were greatly increased numbers of autolysosomes Different possible reasons for this finding are discussed One explanation may be lead induced lysosomal membrane damage leading to leakage of hydrolytic enzymes and cytoplasmic degradation and subsequent formation of autolysosomes This notion is supported by a previously demonstrated lead induced reduction in lysosomal membrane stability using a histochemical method

It is well known that heavy metals are toxic to cells, though the mechanism by which they exert this effect is debated With the ultimate goal of studying the CNS changes in heavy metal poisoning *in vivo*, an attempt was made to elucidate this mechanism *in vitro* on cul tured fibroblasts The present communication deals with the titration of a lead ion dosage suitable for the study, and with the changes caused by the lead, demonstrated in the light and electron microscope

MATERIAL AND METHODS

Culture Conditions

Embryonal rat fibroblasts from minced Sprague Dawley litters were cultured in plastic Petri dishes in Eagle s minimum essential medium supplemented with 10 per cent calf serum and antibiotics The cells were subcultivated at confluence (normally every 5th day) with a split ratio of 1 2 For the experiments cells in phase II (*Hayflick* & *Moorhead*, 1961) from passage 3–6 were used Six hours after subcultivation when the cells had settled a water solution of lead nitrate with 1 μg Pb $(NO_3)_2$/μl was added to the medium in amounts resulting in a concentration of 1, 5, 25, 50 and 100 μg/ml in the cultivation medium The control cultures were untreated The cultures were followed five days

Light Microscopy

Cultures were harvested with a 24 hour interval and fixed *in situ* according to the following scheme the medium was replaced by PBSA to which the same amount of methanol was then added dropwise After a few minutes this mixture was replaced by pure methanol and the cultures were further fixed for 30 minutes After rinsing in glass distilled water they were then stained according to May Grunwald Giemsa, air dried and mounted under coverslips in immersion oil

Electron Microscopy

For the fine structural analysis, cells treated for 1–5 days with 25 μg lead nitrate were fixed in 2

Received 16 i 73 Accepted 29 xi 73

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per cent glutaraldehyde in 0 1 M cacodylate buffer with 0 1 M sucrose (pH 7 4, 510 mOsm) at 0° C for 60 minutes They were postfixed in 2 per cent osmium tetroxide in s collidine buffer (pH 7 3) at room temperature for 90 minutes and dehydrated *in situ* in the plastic dishes in a graded series of ethanol solutions starting with 50 per cent In the last change of 100 per cent ethanol the cells were counterstained with 2 per cent uranyl acetate for 10 minutes

The monolayer was then cut with a razor blade in squares about 2 by 2 mms removed by means of propylene oxide according to *Biberfeld* (1968) further washed in propylene oxide several times to completely remove all remnants of plastic, and embedded in a mixture of Araldite and Epon (No 1) according to *Mollenhauer* (1964) The cell material was spun down in Beem capsules during centrifugation at 10 000 g for 30 minutes A detailed description of the technical procedures has been given previously (*Brunk et al*, 1971)

Ultrathin sections were cut on an LKB Ultrotome stained with lead citrate and examined in a *Siemens Elmiskop 1A* or a *Jeol 100 B TR operated* at 60 or 80 KV

RESULTS

Light Microscopy

In *control cultures* the fibroblasts formed a monolayer covering the bottom of the Petri dish within 36–48 hours Initially after settling the cells were somewhat rounded or elongated, but by day 2 they were uniformly slender with an ovoid nucleus, on day 3 the cells formed whorls and strands (Fig 1), and grew increasingly until forming a confluent dense layer on day 5 There were only occasional cells with nuclear pycnosis and scattered small round cytoplasmic vacuoles during the observation period

1 µg/ml lead did not cause degenerative changes in excess of those described in the control group

5 µg/ml lead did not influence the growth rate of the cultures but caused a slight increase in the number of cells showing cytoplasmic vacuoles These vacuoles were round about 1 µm in diameter and occurred in groups occupying one or more small areas of the cytoplasm These vacuoles showed up from day 3 Some cells in the cultures showed pycnosis and presence of cytoplasmic vacuoles while adjacent cells might have cytoplasmic vacuoles only

10 µg/ml lead caused slightly more pronounced changes than with 5 µg/ml, showing up on day 3 These alterations consisted mainly in the presence of a greater number of cells with pycnosis and cytoplasmic vacuoles occurrence of a somewhat less dense cell layer with small empty spaces and reduced density of the cell population with greater variation in nuclear and cellular size and shape (Fig 2)

25 µg/ml lead caused more widespread changes on day 3 consisting of pycnosis and shrinkage of scattered cells, and occurrence of vacuoles in many cells—often occupying most of the cytoplasm The vacuoles became larger than with the smaller doses, and the cytoplasm turned finely granular towards the end of the cultivation time There also appeared empty spaces in between which the cells varied in shape with either plump or elongated cytoplasm and ovoid to pearshaped sometimes large nucleus (Figs 3 and 4) These changes were seen throughout the cultivation time but increased in intensity towards the end of the period but without death of whole or large parts of the culture

50 µg/ml lead produced marked retardi-

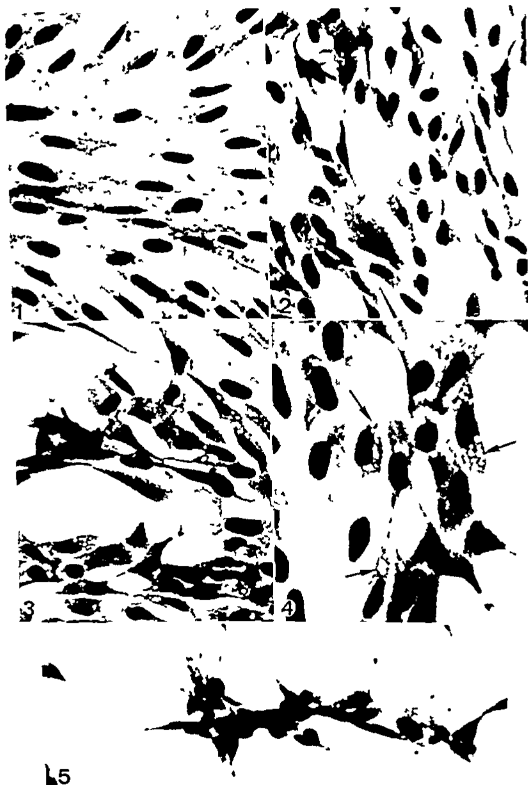
Fig 1 Control culture on cultivation day 3 Uniform cells arranged in strands May Grunwald Giemsa × 300

Fig 2 Culture exposed to 10 µg lead nitrate/ml medium for 3 days Note slight disturbance of orderly arrangement and cellular uniformity Scattered pycnotic nuclei and some empty spaces May Grunwald Giemsa × 300

Fig 3 Culture on day 3 exposed to 25 µg lead nitrate/ml medium Accentuation of changes shown in fig 2 Note increased number of pycnotic nuclei and cells with cytoplasmic vacuoles May Grunwald Giemsa × 400

Fig 4 Detail from fig 3 in higher magnification to show pronounced vacuolization in many cells (arrows) × 800

Fig 5 Culture on day 3 exposed to 50 µg lead nitrate/ml medium Damaged shrunken cells with pycnotic nuclei May-Grunwald Giemsa × 400


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tion of growth already on day 1 and cellular necrosis on day 2 with death of the culture in large areas on day 3 Cytoplasmic vacuolization appeared on day 1 and was increased on day 2 and 3, much in excess of what was seen with lower dosages, although the cellular necrosis presumably obscured many of these changes (Fig 5)

100 µg/ml lead produced a picture similar to that with 50 µg/ml though even more advanced, terminating in complete death of the culture on day 3

Electron Microscopy

The material selected for fine structure analysis was confined to cultures exposed to 25 µg/ml lead The most obvious early changes noticed following exposure to lead occurred within the lysosomal system and the Golgi region The descriptions in the following are accordingly concentrated on these structures

The cytoplasm of the control cells (Fig 6) contained occasional vacuoles and bodies presumed to be secondary lysosomes containing some granular and membrane-like material The Golgi zones were moderately well developed

One day following exposure to lead the cellular structures were not significantly changed as compared to the control cells After two days the Golgi regions seemed somewhat more prominent with wide saccules surrounded by large numbers of small vacuoles, many of which were coated Further, the number of secondary lysosomes containing membranous material appeared to be increased

On day 3 the lead poisoned cells were found to contain large amounts of vacuoles limited by a single membrane and containing membranous material and osmiophilic dense structures and irregular granularities Many of these vacuoles had the appearance of autolysosomes containing material interpreted as more or less completely degraded cell organelles (Figs 7 and 8)

Four days following the initial exposure to lead nitrate the cellular alterations were pronounced with excessive accumulation of autophagic structures (Figs 9 and 10) Further, there was dilatation as well as fragmentation of the endoplasmic net and swelling of the mitochondria These changes were even more pronounced on day 5

Fig 6 Control cells on cultivation day 3 Only occasional secondary lysosomes × 27,000

Figs 7 and 8 Cells exposed to 25 µg lead nitrate/ml medium for 3 days Prominent Golgi apparatus (fig 7) as well as many secondary lysosomes with membranous material and sometimes also electron dense granules (arrow, fig 8) which might indicate lysosomal accumulation of lead × 20 000 (fig 7) and × 28,000 (*fig 8*)

Fig 9 Lead exposed cell on day 4 (same dose as in figs 7 and 8) Heavy accumulation of secondary lysosomes many of which contain myelin figures Well developed Golgi zones × 27,00

DISCUSSION

Lead nitrate in a concentration of 25 µg/ml cultivating medium constantly provoked cellular alterations which were easily demonstrable in the light microscope The changes occurred conveniently within the period of time (6–8 days) during which the strain of fibroblasts used can be kept without subcultivation in a more or less stationary phase (before they start to show normally occuring degenerative changes) The lower dosages tested were unsatisfactory in one or more of the aforementioned respects and higher dosage resulted in rapid death of the cell culture Thus, 25 µg/ml appears to be a border concentration in terms of the cellular tolerance to lead under the present culture conditions This concentration was chosen with the intention to produce changes which would not advance too rapidly but be suitable for an attempt to a fine structural analysis of the mechanism behind the injurious effect of lead

The noxious effect of the lead ions was most clearly expressed by an increasing accumulation of secondary lysosomes some of

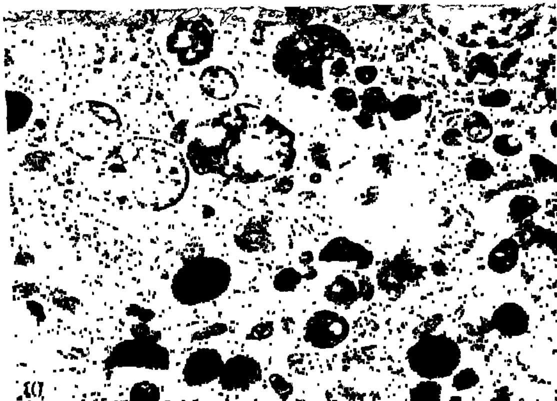


Fig 10 Lead exposed cell on day 4 (same dose as in figs 7 and 8) Advanced alterations with occurrence of many large, membranebound vacuoles Some of these contain electron dense granules of the same type as shown in fig 8 (arrows) × 22,000

which contained rather well preserved cytoplasmic organelles and thus could be autophagosomes or early autolysosomes Signs of advanced cellular damage, such as swollen mitochondria or dilated and fragmented endoplasmic reticulum, were observed only late in the course of the cellular reaction to lead

Concerning the mechanism behind the observed alterations, the described findings might invite to the following intrepretations

(*1*) The accumulation of autolysosomes may be due to enhanced autophagocytosis resulting from unspecific response to general cell damage, known to occur in many situations under toxic influences (*Ericsson* 1969)

(2) The accumulation may, alternatively, be related to inhibition of lysosomal enzymes We have previously shown that lead becomes incorporated into lysosomes when *in vitro* cultured cells are exposed to this metal (*Brunk & Brun* 1972) Since heavy metals are potent inhibitors of several enzyme systems, it lies close at hand to expect depression of lysosomal lytic enzyme activity with consequent slowing down of the degradative processes within lysosomes, including degradation of cellular organelles sequestered in autolysosomes during the normal process of autophagocytosis This would lead to an increase in the number of autolysosomes, without any speeding up of the degree of autophagocytosis [illegible]

of [illegible]

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1972) This is presumably the reason why structurally recognizable cell organelles are rarely seen in autolysosomes If, on the other

hand, the enzyme activities are depressed or inhibited, one would expect to find more of recognizable organelles

(3) A further possibility involves damage of the lysosomal membranes After the uptake of lead, which most likely occurs as a lead protein or a lead amino acid complex, the metal would be set free through lysosomal enzyme digestion of the organic moiety of the complex The released lead may, apart from enzyme inhibition, also damage the surrounding lysosomal membrane, possibly leading to escape of lysosomal enzymes and focal cytoplasmic degradation This might result in sequestration within autophagic vacuoles of the damaged parts of the cell

These hypotheses are all speculative and need further experimental support The third alternative, ascribing the injurious effect of lead to a lysosomal membrane damage, gains indirect support from the histochemical finding of reduced lysosomal membrane stability in *in vitro* cultivated embryonic rat fibroblasts exposed to lead (*Brun* & *Brunk* 1973)

Supported by the *Swedish Medical Research Council* (grant No 12X 2037) and *Svenska Lakaresallskapet*

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Acta path microbiol scand Section A 82, 319–325, 1974

MEDULLARY CARCINOMA OF THE BREAST

Type I and Type III Tumours

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Seventeen cases of infiltrating medullary carcinoma of the breast are presented The majority were of cytological Type I, of poor prognosis Their mean survival time was 2 3 years Five cases of Type III medullary carcinoma are also documented In these cases the patients were all alive over 10 years after operation

In a series of cases of infiltrating mammary carcinoma from the Armed Forces Institute of Pathology 4 3 per cent were found to be of medullary type (*McDivitt et al* 1968) The mean survival time of treatment failures, i e those dying of their disease, was 2 25 yrs, the shortest period of any histological group studied There was however an "almost complete absence of cancer deaths from medullary carcinoma after 5 years' They conclude that if medullary carcinoma is going to prove fatal it will prove fatal early

This finding of a lack of medullary carcinomas that kill after a latent interval of over 5 years was parallelled in the small autopsy series reported from this Institute (*Hartveit* 1971 and 1972) While this series is being extended, it was also felt necessary to examine the material at present available in detail with particular reference to the cytological Type (*Hartveit* 1971) and other histological characteristics of these tumours, to see if it were possible to differentiate medullary tumours that kill from those that are presumably cured by present methods of treatment

Received 12 xi 73 Accepted 3 xii 73

MATERIAL AND METHODS

The material was taken from the files of the Gade Institute, Department of Pathology All were cases of infiltrating breast carcinoma In all cases histology showed them to be medullary carcinomas, according to *Moore & Foote's* (1949) definition The method of cytological typing has been described previously (*Hartveit* 1971) Type I tumours with indistinct tumour cell borders lobulated crowded nuclei and a low nucleo cytoplasmic ratio are of poor prognosis while the opposite characteristics indicate a Type III carcinoma in which a survival time of 10 years or more can be expected The autopsy series for the years 1963–70 contained 13 medullary carcinomas Of these 12 were of cytological Type I Four will be presented in detail, while details of the other 8 will be given briefly in table form The five Type III medullary carcinomas traced over the same interval from our autopsy files or indirectly from our surgical material will be reported in detail Too few Type II medullary carcinomas were found for comparison

Case Reports

Case 1 When she was 56 years old this patient presented with a tumour in her left breast A radical mastectomy was carried out A fig sized tumour which was soft in the centre was present Histology (P 2005/65) showed it to be a Type I medullary carcinoma (Figs 1+2) The tumour cells were of uniform appearance with large vesicular nuclei, and many mitoses were present The lymph nodes were tumour free She was given post-operative

x ray treatment Twenty one months later a tumour mass was discovered on x ray behind the sternum This was treated by x ray and cortico steroids She died 9 months later

Necropsy (0 836/67) showed a tumour infiltrate in the chest wall to the left of the sternum and metastases to the pleura lungs and liver

Case 2 Ten months before her death this 55 year old woman noticed that her right breast had begun to increase in size Five months later a local mastectomy was carried out An ulcerated area (4 cm in diameter) was found in the skin 8 cm from the nipple From this a sinus led into an abscess cavity On section the whole breast consisted of grey nodular tissue Histology (P 3382/63 and P 3757/63) showed a Type I medullary carcinoma (Figs 3+4) The tumour cells which were of uniform appearance with many mitoses formed broad anastomosing cords She was given post operative x ray treatment and later cytostatic treatment Palpable lymph nodes developed in the right axilla two weeks later These increased rapidly in size, a mass the size of an orange being present two months later, when she was also found to have lung metastases She was given cytostatic treatment but died two months later

Necropsy (0 572/63) showed multiple local skin recurrences in the mastectomy scar, tumour masses in the right axilla and multiple lung metastases

Case 3 When she was 49 years old this patient discovered a plum sized tumour in her left breast Enlarged glands were present in the axilla Histology (P 4148/63) showed these contained metastatic tumour A radical mastectomy was carried out Histology (P 4180/63) showed a Type I medullary carcinoma There was marked lymphocyte infiltration of the stroma the tumour cells were of uniform character and showed frequent mitoses A week later bilateral oophorectomy was carried out and she was given post-operative x ray treatment Fifteen months later metastases were found in her left lung She was given steroid therapy and cytostatic drugs Six months later she developed back pain and x ray confirmed the presence of metastases She died two months later, two years after the start of her illness

Necropsy (0 248/65) confirmed the presence of metastases from her breast cancer in the lungs spinal column pituitary and liver

Case 4 Three years before she died this 57 year old woman presented with a mandarin sized tumour in her left breast She was given pre operative x ray treatment Radical mastectomy was then carried out Histology (P 4716/65) showed a Type I medullary carcinoma with metastases to the fatty tissue in the axilla The tumour cells in this case showed a tendency to papillary growth pattern with a delicate connective tissue stroma which showed slight lymphocyte infiltration The tumour cells were of monotonous uniformity with infrequent mitoses She developed skin metastases in the chest wall treated with prednisone A few hours before her death 3 years post-operatively she was admitted with massive ascites

Necropsy (0 285/68) showed multiple skin metastases in the skin of the thorax and the left arm Metastatic tumour was found in the liver peritoneum ovaries Fallopian tubes ureters adrenals the skeletal system lymph node and spleen

Case 13 When she was 48 years old this patient noticed a plum sized lump in her left breast which was removed 3 months later Histology (P 298/50) showed an infiltrating Type III medullary carcinoma (Figs 5+6) The tumour cells grew in broad anastomosing cords separated by connective tissue stroma with little lymphocyte infiltration Within the cords of tumour cells were areas of

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Fig 1 Case I Histology of the tumour (H+E × 112)
Note The uniform tumour cell type and slight lymphocyte infiltration of the stroma

Fig 2 As above (H+E × 448)
Note Tumour cells of Type I Indistinct cell borders lobulated nuclei crowded together and often overlapping

Fig 3 Case 2 Histology of the tumour (H+E × 112)
Note Broad anastomosing cords of tumour cells of uniform type

Fig 4 As above (H+E × 448)
Note Type I cells with indistinct cell borders and lobulated crowded nuclei

Fig 5 Case 13 Histology of the first primary tumour (H+E × 112)
Note The area with Type III tumour cells with clear cell borders and lack of nuclear crowding

Fig 6 As above (H+E × 448)
Note Tumour cells of Type III

Fig 7 Case 15 Histology from the breast tumour (H+E × 112)
Note The lymphocyte infiltrated stroma and Type III tumour cells

Fig 8 As above (H+E × 280)
Note Tumour cells of Type III

Fig 9 Case 16 Histology of the first primary breast tumour (H+E × 112)
Note The tumour cell detail is almost completely obscured by lymphocyte infiltration

Fig 10 As above (H+E × 448)
Note Type III tumour cells are recognisable despite many mitoses

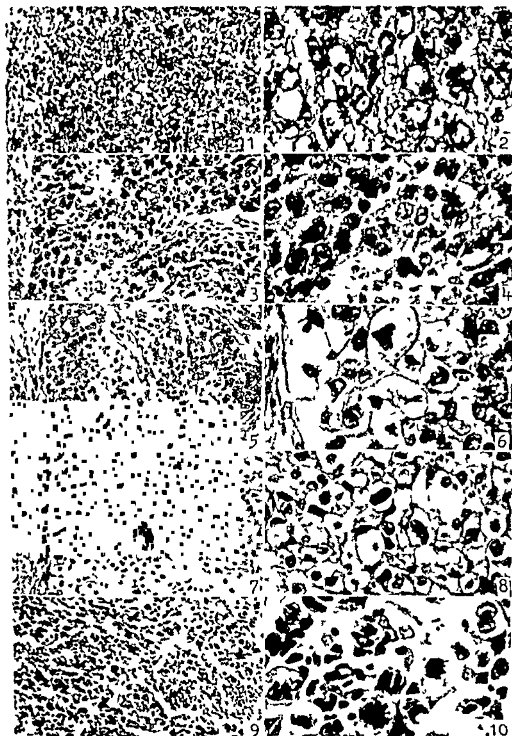

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TABLE 1 *Clinical and Histological Findings in 17 Infiltrating Medullary Carcinomas of the Breast Related to Cytological Type*

	Case No	Cytological Type	Node involvement	Lymphocyte infiltration	Age at operation (yrs)	Survival time (yrs)
1	(2005/65)	I	absent	slight	56	2
2	(3382/63)	I	present	slight	55	1
3	(4148/63)	I	present	marked	49	2
4	(4716/65)	I	present	slight	57	3
5	(4787/68)	I	present	moderate	70	1
6	(4235/66)	I	absent	slight	51	2
7	(8592/66)	I	absent	absent	40	3
8	(4535/65)	I*	absent	slight	76	4
9	(7153/62)	I	absent	slight	73	5
10	(4323/66)	I*	absent	slight	29†	1
11	(10059/65)	I	present	slight	54	1
12	(3865/61)	I*	present	sl ght	45	3
13	(298/50)	III	absent clinically	absent	48	13
14	(304/48)	III	absent	moderate	42	15+
15	(4348/54)	III	absent	moderate	59	14
16	(205/41)	III	absent	marked	29†	33+
17	(1339/39)	III	absent clinically	moderate	31	24

* some definition of tumour cell borders
† tumour discovered in puerperium

Type III cells with clear cell borders, a lack of nuclear crowding and nuclear lobulation, but with considerable variation in nuclear size and stain ability The lymph nodes were clinically tumour free and were not removed She was well for the next 12 years when she noticed secretion from her right nipple Two months later she had a firm tumour mass in her right breast lung and lymph node metastases She was treated with local x ray and later cortisone She died four months later

At necropsy (0 388/63) she was found to have a scar from her left local mastectomy, and a firm hen s egg sized tumour in her right breast This was considered to be a second primary, Type I Metastatic tumour was also found in lymph nodes lungs pleura liver and spinal column

Case 14 When she was 42 years old this woman noticed a lump in her right breast Two months later a radical mastectomy was carried out Histology (P 304/48) showed a Type III medullary carcinoma The histological picture was very similar to that in Case 13 but the stroma showed mode rate lymphocyte infiltration The lymph nodes were tumour free She was given post operative x ray treatment She was well for the next 15 years when she found a plum sized tumour in her left breast She was also found to have enlarged glands in her left axilla and left supra-clavicular fossa A local mastectomy was carried out Histology (P 8702/63) showed an infiltrating scirrhous carcinoma (Type I) She was again given post operative x ray treat ment and also x ray castration Metastases in the left mastectomy scar removed the following year showed a similar histological picture She has not been traced since

Case 15 When she was 59 years old this patient had a radical mastectomy for a right sided breast tumour Histology (P 4348/54) showed a *Type III* medullary carcinoma (Figs 7+8) In this tumour the stroma that showed moderate lymphocyte infil tration, was well demarcated from broad *cords* of Type III tumour cells These were of uniform appearance but again showed considerable *varia* tion in nuclear size and stainabil ty The lymph nodes were tumour free She was given post-opera tive x ray treatment She was well *until a year* before her death at 83 years age when she deve loped haematuria A papillomatous tumour was found in the bladder Histology (P 10081/68) showed a primary bladder cancer A palliative transurethral resection was carried out She d ed 10 days later

Necropsy (0 978/68) showed the cause of death to be massive bilateral lung emboli Her blad ler

carcinoma infiltrated the bladder wall and occluded the left ureter There were no distant metastases and no evidence of recurrence of her breast cancer

Case 16 At 29 years of age, 6 weeks after the birth of her second child this patient noticed a tumour in her right breast The tumour was removed (P 205/41) and later radical mastectomy was carried out (P 255/41) A Type III medullary carcinoma was found (Figs 9+10) This tumour showed gross lymphocyte infiltration, great variation in nuclear size and very frequent mitoses The lymph nodes did not contain tumour The breast tissue showed fibroadenomatosis with apocrine cysts She was given post operative x ray treatment Three years later a papillomatous cyst in the right ovary was removed Seven years after that a papillomatous cyst adenoma with some areas of atypical epithelial proliferation was found in the left ovary which was also removed There was no sign of infiltrative growth. Nineteen years after the removal of the cancer in the right breast she presented with a Type II primary tumour in her left breast (P 5969/60) This had been present for about two years Radical mastectomy was carried out (P 6145/60) The lymph nodes were tumour free Curettings from the uterus at this time showed follicular hyperplasia Three years after this she developed a tumour nodule in the left side of the thorax (P 2828/63) which was treated by irradiation A further nodule in the chest wall under the left mastectomy scar (P 3089/67) was removed 4 years later

At the age of 42 years she was found to have bilateral lung tuberculosis (apical) and has been under treatment for this She also has extensive bronchiectasis and pulmonary fibrosis For the last year and a half she has had repeated small haemoptyses and dyspnoea on exertion Repeated investigation for metastatic tumour in the lungs including bronchoscopy and exfoliative cytology has been negative She is clinically tumour free at the time of writing

Case 17 When she was 31 years old this patient noticed a tumour in her right breast which increased rapidly in size and was removed 3 months later Histology showed a Type III medullary carcinoma with moderate lymphocyte infiltration of the stroma The axillary nodes were clinically tumour free She was given post-operative x ray treatment Twenty three years later she was admitted with an enlarged spleen The diagnosis of chronic myeloid leukemia was made She was treated with prednisone and died of pneumonia 6 months later

The diagnosis was confirmed at autopsy (O 144 63) No recurrence of her breast carcinoma was found

The cases described above demonstrate the poor prognosis of Type I medullary carcinoma of the breast in contrast to Type III Table [illegible] gives a summary of the findings in these cases and those in the additional cases recovered from our records

Node involvement was present in half of the Type I tumours, but was uniformly absent in the Type III The degree of lymphocyte infiltration varied in both types, but was perhaps more marked in Type III The age at operation varied in both types but the survival time was markedly different mean 2 3 years in Type I and over 10 years in Type III

It is of note that a second breast primary occurred in Cases 13, 14 and 16, while Case 15 died of carcinoma of the bladder and Case 17 of chronic myeloid leukemia

DISCUSSION

The histological diagnosis of medullary carcinoma of the breast has been discussed in detail by *Moore & Foote* (1949) They stress that both the macroscopic and microscopic appearance of these tumours is characteristic, and that they are less malignant than might be supposed from their degree of cytological atypism In the cases in the present series the macroscopic descriptions of the tumours were not detailed enough to help in classification But the histological appearance conformed to that described and illustrated by *Moore & Foote* (1949) They believe this to be a readily recognisable histological type, and qualify the term by adding "with lymphoid infiltration" were applicable Microscopically, while a certain papillary quality may be present, the tumour usually consists of broad anastomosing masses of cells of rather uniform character with numerous mitoses They note that the nuclei do not show great variation in hyperchromatism, size or shape

This description is repeated by *McDivitt et al* (1968) Even so they remark that medullary carcinomas of the breast are often markedly anaplastic This paradox has caused great difficulties with prognostic grouping and it has even been suggested (*Gorski et al* 1968) that they should be put into the group of best prognosis irrespective of their histological appearance

The incidence of medullary carcinoma in the series from which the present cases were drawn was 14 9 per cent that is higher than

that of the Armed Forces Institute's series but nearer to the 10 2 per cent in the surgical material from this Institute (*Mæhle & Hartveit* 1973) The survival time of treatment failures was however similar, 2 3 compared to 2 25 years These cases in the present series were of cytological Type I No Type III medullary carcinomas were found among the treatment failures

The five Type III tumours were found among patients dying of other causes, or presenting later with bilateral disease They all lacked spread to the axillary nodes at the time of operation, in contrast to Type I, but like Type I they occurred at a variety of ages All five were characterized by a uniformly good prognosis The prolonged survival time after operation may indicate that these tumours present locally before spread has occurred or that they are so slow growing that spread is still occult up to 30 years after operation All these patients however developed a second malignancy from which 3 have since died It may be that they merely lived long enough to develop a second cancer (*Robbins & Berg* 1964, *Cady* 1970), but the possible presence of other factors predisposing them to malignant disease breast cancer in particular, should not be overlooked The association between medullary carcinoma and apocrine metaplasia may be pertinent here (*Hartveit* 1973b), while Case 16 illustrates the possible results of hormonal imbalance

The presence of lymphocyte infiltration is said to be of good prognosis in medullary carcinoma in general (*Moore & Foote* 1949) This is substantiated in the present small series in which infiltration was extensive in 4 out of 5 Type III carcinomas, compared to 2 out of 12 Type I As reported previously (*Hartveit* 1973a) it does not appear to influence prognosis within cytological Type I

The histological findings in the Type I carcinomas in the present series are in keeping with *Moore & Foote s* (1949) description of broad anastomosing masses of cells of rather uniform character with numerous mitoses Such cells are anaplastic in the sense that they appear to be un- or de-differentiated, in keeping with the "embryonal look described by *McDivitt et al* (1968) In some of the Type I carcinomas slight definition of the cell borders was seen in some areas but this was not sufficiently marked to lead to a Type II diagnosis Even so it may cause confusion However, in these cases it was accompanied by Type I nuclear characteristics, and crowded lobulated nuclei that were of uniform size and shape

In contrast the cells of Type III medullary carcinomas, with their well defined cell borders, abundance of cytoplasm and smooth nuclear outline showed great variation in nuclear staining nuclear size and shape They too can well be described as of anaplastic appearance In spite of this they belong to cytological Type III, in which a long survival time can be expected, as the criteria used in cytological typing of infiltrating breast carcinomas are not those of anaplasia, as defined by *Hansemann* (1893) as the degree of divergence from the cell of origin For example variation in nuclear size and staining are not used Further the presence or absence of mitotic figures is not a determining factor Here perhaps lies the root of *Gorski et al's* (1968) difficulty Their material may have contained Type III rather than Type I medullary carcinomas The latter appear to correspond to *McDivitt et al s* (1968) medullary carcinomas of poor prognosis

This work was carried out with the technical help of Miss *Sidsel Heieren* financed by the *Norwegian Cancer Society*

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Acta path microbiol scand Section A 82, 326 336, 1974

INTRACRANIAL TUMOURS INDUCED IN GUINEA PIGS WITH ROUS SARCOMA VIRUS

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Fifty newborn guinea pigs were inoculated intracranially with Rous Sarcoma virus strain Schmidt Ruppin (SR RSV) Neurological symptoms appeared after 3–7 weeks Forty eight intracranial tumours were found in 27 guinea pigs Ten guinea pigs showed meningeal tumours in 10 guinea pigs the tumours were located intracerebrally and 7 showed both meningeal and intracerebral tumours The meningeal tumours were either highly differentiated fibroma like often growing in whorl like formations and sometimes invading the overlying skull bone or sarcomatous Similar sarcomas with both argentophilic and glial fibrils were seen among the intracerebral tumours The 'mixed character may be due to an inherent capacity of the tumour cells to produce both argentophilic and glial fibrils Nine of the intracerebral tumours had the appearance of gliomas resembling the fibrillary astrocytomas or oligodendrocytomas in man Angiographically arteriovenous shunts were seen in the sarcomatous intracranial tumours whereas displacement of the vessels was nearly absent in the poorly vascularized gliomas The mengiomas received their blood supply from the external carotid artery although it would be supplied from the internal carotid artery if they had an intracerebral component

Intracranial neoplasms of different types have been induced with virus leptomeningeal tumours in Syrian hamsters (15) and rabbits (10) by polyoma virus papillary ependymonas in Mastomys (17) and Syrian hamsters (9) by *Simian vacuolating virus* (SV 40) and undifferentiated malignant *tumours in mice and Mastomys by adeno* virus 12 (16) In all experiments, new born *animals were used and the virus was usually* inoculated intracerebrally sometimes sub*cutaneously*

Rous sarcoma virus (RSV) inoculated into the brain of fowls produces sarcomas of the same myxosarcomatous type as after subcutaneous or intramuscular injection (20) In mammals other types of intracranial tumours are obtained RSV strain Schmidt-Ruppin (SR-RSV) and strain Bryan induce gliomas in hamsters (13 14 4) and leptomeningeal sarcomas as well as gliomas in dogs (12 8) after intracerebral injection *Bigner et al* (2) obtained gliomas in about two thirds of the SR RSV inoculated dogs and recognized almost all of the glial tumour types observed in man the exception being oligodendrogliomas and medulloblastomas *Zimmerman* (23) considered the canine tumours induced by *Rabotti et al* (12) to be sarcomas and not gliomas but electron

Received 20 vii 73 Accepted 30 xi 73
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microscopic evidence of the glial origin of some of the tumours has been provided (21) SR RSV has induced intracerebral tumours in primates (19)

SR RSV is oncogenic in guinea pigs after intramuscular injection (1) The aim of the present investigation was to elucidate the oncogenic capacity of SR-RSV in guinea pigs after *intracranial* inoculation, especially with regard to the capacity of the virus to affect the glial cells

Another aim was to study the vasculature of the intracranial tumours by means of angiography In man, different kinds of tumours have a characteristic angio archi tecture which facilitates establishment of a specific diagnosis of the tumour type Malig nant gliomas usually contain AV shunts and thus are revealed by early venous filling Astrocytomas, on the other hand, are often poorly vascularized They may also infiltrate, making diagnosis difficult, since no large mass can be seen This is in contrast to cancer metastases which often are surrounded by a pronounced oedema causing an expansion greater than the tumour per se Meningiomas are mainly supplied by the external carotid artery, contrary to tumours originating from the true brain parenchyma They are often richly vascularized but seldom contain AV shunts

MATERIAL AND METHODS

The Schmidt Ruppin strain of Rous sarcoma virus (SR RSV) was used in the form of a virus pool obtained from an homogenate of rapidly growing chicken sarcoma purified by repeated ultracentri fugations and kept in saline at –70° C The virus titre was 5 × 10 FFU tested accord ng to Tem n and Rubin Forty one animals were injected with the pool In addition 9 guinea pigs were inoculated with a finely minced suspension of chicken wing web sarcoma in saline 1 5 with 0 10 mg of strepto mycin and 0 10 mg chloromycetin per ml

Random bred guinea p gs of both sexes were used most of them 7 12 days old three 1 day old and five 14 days old The we ght varied between 90–180 gr They were kept on standard pellet diet with additional fresh greens

The guinea pigs were inoculated in the mid portion of the left cerebral hemisphere at a depth of about 3 mm us ng a 16 gauge needle To pre vent the needle from being inserted too deeply it was surrounded by a plastic tube leaving the distal 4 mm of the needle free The animals were given one 0 1 ml injection of the virus pool on three consecutive days whereas only one injection of 0 1 ml of the sarcoma suspension was given

The guinea pigs were exam ned once a week Nine of the pool injected animals died within 2 weeks after the injection Sixteen guinea pigs died or were killed when moribund after 4–8 weeks The remaining animals were killed with ether 12–20 weeks after the injection

Autops es were performed on all animals Usu ally 5 per cent formaldehyde was slowly injected through the carotids or the head was kept in 5 per cent formaldehyde for 48 hours before the skull was opened

The brain was cut in thin coronal sections All brains with tumours were photographed

Specimens of the tumours were embedded in [illegible]

tion according to Gridley or Gordon Sweet to demonstrate reticulin

Arteriography was performed on 8 tumour bearing guinea pigs General anaesthes a was ob tained by pentobarbitone sodium (Mebumalnatri um ACO, Sweden) intraperitoneally The upper abdomen was opened by a high midline incision The descending aorta was cannulated cranially and the infer or caval vein opened The vessels in the upper half of the animal were rinsed by injection of physiological saline with heparin A fine sus pension of barium sulphate (Micropaque Da mancy & Co Ltd , England) with some gelatin dissolved in hot water was then slowly injected through the cannula into the aorta The injection pressure was controlled by an electromanometer since it would otherwise often climb too high (> 300 mm Hg) causing rupture of the vessels The filling of the blood vessels in the head was controlled during magnification fluoroscopy and after termination of the injection roentgen films in antero-posterior and lateral projections were taken The animal was then cooled in a refrigerator for about four hours to set the gelatin Autopsy was performed and the brain dissected free A pair of roentgen films of the brain was taken Technical data 1 0 mm focal spot FFD 90 cm Agfa Gevaert industrial film D4 32 mAs and 85 kv

It is perfectly possible to perform angiography *in vivo* by selective inject on of the contrast medi um into the internal or the external carotid artery or into the vertebral artery The an mals were however usually in a poor condition due to the tumours and in the few cases where *in vivo* studies were attempted the an mal died before the procedure could be completed The gamma camera techn que of study ng bra n tumours s much less

traumatic but the resolution obtained is not very good especially when the structures are as small as those in the guinea pig brain

RESULTS

Neurological symptoms appeared in 14 animals 3–7 weeks after inoculation The guinea pigs often became somnolent and developed ataxia, circling and unilateral sometimes bilateral paresis of the hind legs The head was often tilted In the final stage, the animals were often lying motionless with shallow breathing and trembling of the hind legs Six animals were found dead without preceding neurological symptoms Extensive but neurologically silent intracerebral tumours were found in 2 guinea pigs killed 14 and 16 weeks after the inoculation No tumours were found in the 9 guinea pigs which died within two weeks after the injection The cause of death was meningitis in three of them whereas the other showed bronchitis or focal pneumonias

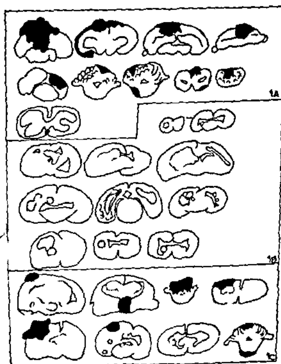


Fig 1 The location of the tumours in 27 guinea pigs after intracranial injection of SR RSV A Meningeal tumours B Cerebral tumours C Meningeal and cerebral tumours





Fig 2 a Meningeal tumour invading the skull bone and bulging into the cranial cavity, distinctly demarcated from the brain

Fig 2 b Osteomatous meningioma causing a cup like depression in the underlying hemisphere A subependymal tumour is visible in the right hemisphere

Tumours appeared in 27 of the 50 guinea pigs namely in 18 of the 41 animals injected with the cell-free virus pool and in all of the 9 guinea pigs injected with cellular suspension of the Rous chicken sarcoma The location of the tumours is schematically given in Fig 1 Ten guinea pigs showed tumours in connection with the meninges compressing and in two cases also invading the underlying brain and sometimes also involving the overlying skull bone (Fig 1 A) These are hereby designated as meningeal tumours Ten guinea pigs showed tumours in the cerebrum without visible connection with the cerebral coverings (Fig 1 B) They are designated as cerebral tumours Seven guinea pigs showed both meningeal and cerebral tumours (Fig 1 C) Eleven guinea pigs had multiple tumours In all 48 tumours were seen Among the 9 guinea pigs injected with the cellular suspension 4 developed cerebral

tumours and 5 both cerebral and meningeal tumours

Hydrocephalus was sometimes seen, especially in brains with subependymal tumours

1 *Meningeal Tumours*

They were usually located on the convexity of the brain at the site of injection (Fig 2 a) or between the hemispheres and the cerebellum, sometimes also on the under surface of the brain The tumours had a greyish white colour and were intimately connected with the meninges, bulging into the cranial cavity, sometimes also extending laterally over the surface of the brain The tumours were usually distinctly demarcated from the brain and covered by a protective pia, but invaded in three cases the underlying brain tissue In 4 animals, the overlying bone was involved and, at the time of death, 3 of these showed a hard round tumour located in the parietal bone in the region where the skull was penetrated by the injection needle, sometimes causing a cup-like depression in the underlying hemisphere and a protuberance on the outside of the skull (Fig 2 b)

Angiographically two meningeal tumours were seen, both of which infiltrated the brain The meningeal component per se was poorly vascularized, received its blood supply from the external carotid artery and in one case resembled an osteoma in its angiographic appearance The infiltration in the brain was richly vascularized, contained shunts, and was supplied by the internal carotid artery The angiographic appearance of the tumour component in the brain was similar to that of a malignant glioma No calcifications were seen in this tumour component

Microscopically the meningeal tumours were mainly of two types The first was highly differentiated and composed of interlacing bundles of elongated fibroblasts with a narrow or rod shaped nucleus and scanty cytoplasm (Fig 3) Numerous reticular and collagen fibres were seen between the cells An obvious finding was the tendency of the tumour cells to be arranged in whorls or in concentrically arranged layers (Fig 4) Often



Fig 3 Fibromatous meningioma Htx eosin × 160

Fig 4 Fibromatous meningioma with a whorl like arrangement of the tumour cells Htx eosin × 400

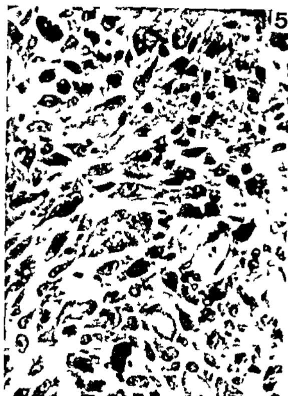


Fig 5 Meningioma of sarcomatous type Htx eosin × 400

the core of the whorl was found to be a small blood vessel with a narrow or obliterated lumen but sometimes the core consisted of concentrically layered thin cells Calcifications in the centre of the whorls were not seen

Single bone trabecules sometimes extended from the overlying skull bone into the tumour Three tumours were almost in toto composed of a plexiform network of osteoid bone trabecules surrounding groups of tumour cells and single multinuclear osteoclasts Numerous sometimes wide, vessels were also visible inside the tumour The intimate connection in these tumours between the tumour cells and the osteoid trabecules favours the opinion that the formation of bone was due to the activity of the tumour cells and not only to non specific periosteal reaction to the tumour growth

The other type (Fig 5) of the superficial tumours was anaplastic and made up of cells with a round, oval or irregular nucleus with rather coarse chromatin and a distinct nucleolus The cells had a smooth outline and usually a sparse, slightly basophilic cytoplasm They were closely packed but on silver impregnation fine reticular fibrils were seen between them Mitoses were common Sometimes one and the same tumour showed some areas of the highly differentiated type and others of the anaplastic, sarcomatous type Focal sharply demarcated necroses were seen in both types of tumours

It is of interest to note that on PTAH stained specimens glial fibrils were found in some of the meningeal tumours and this not only in the vicinity of the underlying brain surface but also in the inner portions of the tumours The fibrils were scanty in the highly differentiated tumours, being more numerous in the sarcomas



Fig 6 a Intracerebral tumour in the left hemisphere bulging into the ventricle

Fig 6 b Fibrillary astrocytoma with a greyish white cut surface Guinea pig 5 months after the injection of a suspension of SR RSV induced chicken tumour

2 *Cerebral Tumours*

The cerebral tumours were located inside the brain and surrounded by brain substance or bulged into the cerebral ventricles (Fig 6 a) The subependymal zones were sites of predilection In two guinea pigs the tumour grew as a nodular layer alongside the ventricular walls Most of the tumours were found in the injected hemisphere, but sometimes in the contralateral hemisphere In subependymal tumours clusters of tumour cells could be seen floating in the ventricles or attached as small polyps to the ventricular walls The colour of the tumours was greyish white Some of them showed haemorrhages or cysts The consistency was usually soft, sometimes, however, it might be rather hard with a glistening cut surface (Fig 6 b)

By microscopy, 9 of the intracerebral tumours were seen to have the appearance of gliomas showing numerous glial fibrils that were stainable with PTAH but not visible on silver impregnated specimens (Fig 7) The fibrils were often clearly located inside the cytoplasm The tumour cells usually had a gemistocyte like appearance with a plump, sometimes triangular or tadpole like shape, rather abundant cytoplasm, and an excentrically located nucleus The admixture of smaller cells with small round nuclei gave the tumour a pleomorphic appearance Another type of glial tumour was composed only of small cells with round nuclei and sparse cytoplasm Delicate glial fibrils were seen between the cells (Fig 8) Three of the gliomas had this appearance which distinctly deviated from the pleomorphic glioma described above

The other type of intracerebral tumour had a sarcomatous appearance similar to the anaplastic type of the meningeal tumours and composed of cells with oval or sometimes slightly indented nuclei and a usually sparce cytoplasm The subependymal tumours often showed rather plump cells with a round or irregular nucleus sometimes excentrically located Argentophile and PTAH positive fibrils were seen inside the tumours Usually the glial fibrils were delicate whereas the

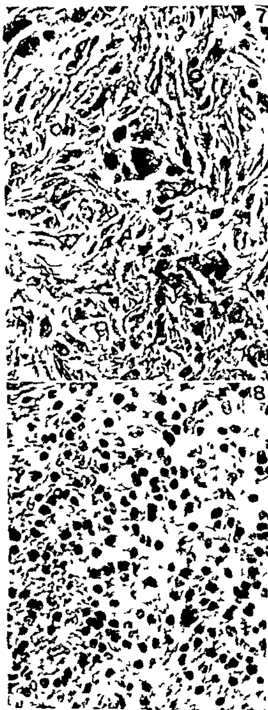


Fig 7 Fibrillary astrocytoma with numerous glial fibrils PTAH according to Mallory × 400

Fig 8 Oligodendrocytoma like tumour composed of small cells with round nuclei and delicate glial fibrils PTAH according to Mallory × 400



Fig 9 Cerebral angiogram Slight bulging of the pericallosal artery (◊) to the right as a sign of a glioma in the left hemisphere, containing some tumour vessels (◆)

argentophile fibrils were coarser, often separating groups of tumour cells The collagenous and reticular fibrils were partly of stromatous origin but partly so intimately related to the tumour cells that they as the glial fibrils must be considered to be derived from these

Four guinea pigs with cerebral tumours were studied by angiography One fibrillary astrocytoma was growing subependymally and nothing but a slight hydrocephalus could be seen on the angiogram Another of the tumours which was also poorly vascularized was located so as to occlude the outlet from one of the ventricles resulting in an unilateral hydrocephalus (Fig 10) One animal had a large, poorly vascularized glioma in the left hemisphere without AV shunts (Fig 9) In one guinea pig a richly vascularized gliosarcomatous tumour with AV-shunts was seen in the left hemisphere (Fig 11)

DISCUSSION

Tumours appeared in *all* guinea pigs inoculated intracerebrally with a suspension of SR RSV chicken sarcoma but in only about 60 per cent of the guinea pigs injected with the cell free virus pool This may be due to quantitative differences in the injected oncogenic dose (3), but may also be due to a temporary survival of the transferred chicken cells with production of infectious virus and a more protracted oncogenic effect That

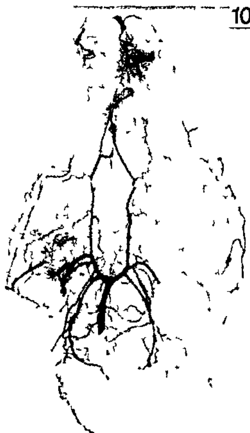


Fig 10 Cerebral angiogram Submentovertical projection A large expanding mass is seen in the right hemisphere No abnormal vessels At autopsy a subependymal gliomatous tumour was found to occlude the outlet from the right lateral ventricle which was dilated



Fig 11 Cerebral angiogram Submentovertical projection A richly vascularized gliosarcoma (⬇) in the left hemisphere AV shunting is seen Due to the high injection pressure a vascular rupture occurred on the other side (⇩)

Rous chicken sarcoma cells can survive for a few days in the brain of an alien host has been shown by *Greene* (7) who transplanted Rous chicken sarcoma to the brains of rabbits guinea pigs and mice, the incidence of takes exceeding 85 per cent Growth was rapid but shortlived and invariably terminated in regression within 2 weeks of transfer

The tumours to appear after the injection of living chicken sarcoma cells had the same microscopic character as the tumours induced by the virus suspension and it is well documented from numerous earlier experiments that mammalian tumours which are induced by Rous chicken sarcoma cells are composed of mammalian cells and not of chicken cells No viral particles could be found by electron microscopic analysis (kindly performed by assoc prof *L G Lindberg*) of one of the meningeal guinea pig tumours 6 weeks after the inoculation with virus pool material, which differs from findings in dogs (6 11) The presence of RSV genome was however demonstrated by the appearance of typical Rous sarcomas in chickens injected in the wing webs with a cellular suspension from the same tumour

It is tempting to compare the *meningeal tumours* obtained in the guinea pigs with the corresponding human neoplasm Both have a multifaceted morphology with several variants ranging from highly differentiated fibroblastic to sarcomatous types The fibroblastic type of the human meningioma is comparable to the differentiated type among the guinea pig tumours The formation of whorls is the most conspicuous character of the human meningeomas and is considered to be an inherent tendency of the meningioma cells Whorls were also present in the fibrous meningeal guinea pig tumours most often arranged around blood vessels but sometimes also around concentrically layered degenerating cells Calcium deposits in the core of the whorls were not seen but this might be due to the relatively short life span of the tumour bearing animals Growth in whorl like formations was not seen in Rous guinea pig tumours induced by intramuscular injection of RSV (1) Coarsely fibred or lamellar bone may be found in human meningiomas of all types This is by some authors considered to be due to an osteoblastic activity of the meningeal tumour cells themselves and by others to be a response of osteoblastic cells in the neighbourhood The same applies to the guinea pig tumours In some of the latter the bone trabecules most probably were a non specific endosteal and periosteal reaction whereas in others a network of bone trabecules was present throughout almost the whole tumour and intimately related to the tumour cells indicating that the bone was produced by these

The anaplastic type of the meningeal guinea pig tumours may be equivalent to the sarcomatous type of the human meningeoma. The occurrence of single glial fibrils in the *meningeal guinea pig tumour can be due to* a malignant transformation of glial cells deposited in the meninges during the injection of the oncogenic agent but may also be due to an *inherent capacity of the tumour* cells. Also in human meningotheliomas some fibroglia may be demonstrated and the cells of origin of meningiomas probably arise from the neutral crest as does the neuroglia (for ref see 5).

Radiologically, meningiomas in human subjects are often seen to involve the bone which increases in thickness and density. Similar changes were noted in the guinea pig, the appearance of the tumour sometimes resembling that of an osteoma. The vascularization of meningiomas in the guinea pig was poor as compared to that in human meningiomas. It should, however, be noted that water soluble roentgen contrast media are used for cerebral angiography in human subjects, whereas the guinea pig angiomas were made with barium sulphate suspension. During the capillary phase of an angiogram made with water soluble contrast medium the meningioma appears very dense, but this is only in part an index of the number of capillaries. There is no blood brain barrier in the meningioma and therefore a considerable amount of contrast medium can leak out through the capillary wall and opacify the tumour. The extent of vascularization may thus be overestimated in human meningiomas. The cerebral component of the meningioma in the guinea pigs was richly vascularized and supplied from the internal carotid artery. A similar blood supply may occur now and then in human meningiomas but it is usually not so extensive as that in guinea pigs.

Intracerebral tumours occurred in 17 of the inoculated guinea pigs but only 9 of the tumours had the character of a pure glioma. Two types of gliomas were seen one was polymorphocellular and resembled a fibrillary astrocytoma with numerous gemistocyte-like cells of varying shape and numerous glial fibrils. The other type was monomorph and similar to human oligodendrocytoma, however, no boxing was observed. Sometimes one and the same tumour had in one area an oligodendrocytoma like character whereas other areas showed the picture of a fibrillary astrocytoma. The glial fibrils were distinctly stained by PTAH and were often seen to lie inside the cytoplasm of the tumour cells. The relative paucity of the pure gliomas obtained *in the guinea pigs contrasts with the findings* by *Bigner* (2 4) who obtained gliomas in two thirds of his Rous virus inoculated dogs and recognized among these almost all of the glial tumour types observed in man.

It has been emphasized by *Zimmerman* (22) that very few experimental gliomas are "pure" tumours composed of cells of one type but rather a mixture of cells of several different types. Many of the cerebral tumours in the guinea pigs contained both mesodermal and glial cells indicated by the presence of both glial and argentophilic fibrils in the tumours. In some of these, the argentophilic fibrils dominated, in others they were arranged in relatively wide meshes whereas the glial fibres were delicate and arranged in close contact with the single cells. The mixed character may be due to a malignant transformation by the Rous sarcoma virus not only of glial cells but also of fibroblasts in relation to the blood vessels at the site of the intracerebral inoculation. The morphological character of the final tumour may depend on the proliferative capacity of the transformed cells either the glial cells or the fibroblasts may win in the mutual competition or both may survive as components of the tumour. It is also possible that the mixed character with the production of glial and argenophilic fibrils is due to an inherent capacity of the affected cells to produce both glial and reticular fibrils. These guinea pig tumours can perhaps be compared to the sarcomas in the human nervous system which are composed both of glial cells and of mesodermal cells (for ref see 18).

The angiographic appearance of cerebral tumours in the guinea pig was similar to that seen in man, ranging from extremely poorly vascularized tumours of the astrocytoma type to richly vascularized tumours containing AV-shunts of malignant glioma type The cerebral tumours in the guinea pig were often extremely difficult to diagnose angiographically like astrocytomas in man The absence of major angiographic signs in some of the animals may also have been due to the small size of the structures

It is difficult to decide whether or not the intracranial guinea pig tumours induced by SR RSV always will grow progressively Lymphocytes and sparse plasma cells were sometimes seen in the periphery of the meningeal tumours and along the ependymal border of some of the intracerebral tumours which might indicate an immunological defence The finding of extensive intracerebral tumours in guinea pigs killed 30–40 weeks after the injection of virus material indicate a persistence and a progressive growth of the induced tumours

The study was supported by a grant from *John och Augusta Perssons Stiftelse for Medicinsk forskning* The technical assistance of mrs *Hrefna Kjartansdottir* is gratefully acknowledged

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Acta path microbiol scand Section A 82, 337-344 1974

AUTOLYTIC CHANGES IN THE HUMAN MYOCARDIUM

Particularly with a View to Detecting Acute Myocardial Infarction by the Nitro BT Method

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Very few studies are available on autolytic changes in the human myocardium We therefore made a study of 30 hearts maintained *in situ* Fifteen hearts were studied on an average 9½ hours postmortem and the other 15 hearts on an average 74 hours postmortem Two myocardial slices from each heart were incubated for 30 minutes at 37° C in a solution consisting of substrate Nitro-BT and buffer Twenty five hearts were intensely and uniformly blackish blue in appearance, due to the formation of diformazan in the myocardium whereas five hearts presented unstained areas These areas were evaluated as being recent myocardial infarcts Micro-enzymatic studies of the same hearts revealed corresponding results With increasing postmortem intervals the diformazan appeared as coarse uneven and disarranged granules With regard to the commonly accepted parameters pertaining to recent myocardial infarct, histological study showed these parameters to vary so much from one heart to another and from one area to another within the individual heart that we abandoned quantitative analysis of these changes The macro- and micro-enzymatic dehydrogenase reaction is well suited for the detection of acute myocardial infarction in all cases up to and including the third postmortem day

Very few studies are available on autolytic changes in the human myocardium *Nicolas et al* (1969) examined 15 hearts of patients without clinico-pathological cardiac changes Portions of the myocardium were maintained in serum at room temperature under sterile conditions Conventional microscopic examination of haematoxylin eosin stained tissue sections revealed no changes within 6 hours after death After 6–12 hours, anisocaryosis had developed pyknosis and hyperchromasia of the nuclei had started After 12–18 hours, partial fragmentation of the muscle fibres was seen, and after 48 hours the fragmentation had become generalized After 3 days the longitudinal and transverse striation was reduced or had disappeared completely, and the muscle fibres were homogenized At the same time the architectural pattern was disrupted After 6 days the nuclei had disappeared

The possible effect of autolysis on enzyme-histochemical reactions as a means of detecting experimental myocardial infarctions was studied systematically by *Kent* (1957) and *Buss* (1970) *Kössling et al* (1972) made a systematic study of smaller sections of non-infarcted myocardium from 9 pigs and 6 human beings for up to 48 hours postmortem

Received 3 x 73 Accepted 1 xii 73

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Apart from this we do not know of any systematic studies of the human myocardium with respect to the possible effect of autolysis in the detection of infarction by macroscopic and microscopic enzymatic dehydrogenase reactions On the other hand, several reports on macro enzymatic detection of infarction provide more conclusive data on the postmortem interval during which the method might be applicable, but the size of the material and/or methodology of examination are not stated Using the tetrazolium salt TTC, *Walther et al* (1969) examined 5 human hearts and found that the method had to be used early and at any rate within 48 hours postmortem *Knight* (1967) reported favourable results up to 36 hours postmortem and even later *Nachlas & Shnitka* (1963) who used the tetrazolium salt Nitro-BT, found that this method could be employed as late as 48 hours postmortem if the body was maintained at 4° C, but only for 8 hours at 25–37° C and with the heart *in situ* *Ramkisson* (1966) also found the method applicable for up to 48 hours if the body was stored at 6° C, whereas *Kalderon* (1968) did not state any time limit, but was of the opinion that the macroscopic method was of limited value only

Therefore, we decided to carry out autolytic studies on human hearts maintained *in situ*, particularly with a view to the macroscopic applicability of the Nitro BT method when detecting acute myocardial infarction At the same time we wished to evaluate any possible obscuration by autolysis of generally accepted histological infarction changes

MATERIAL AND METHODS

The material was 30 hearts maintained *in situ* In our hospital bodies are for the first 6 12 hours stored at 16° C and subsequently at 4° C Fifteen hearts were studied within 12 hours (7–12 hours) on an average within a period of 9½ hours and the other 15 hearts more than 60 hours postmortem (60–111 hours), on an average of 74 hours Apart

TABLE 1 *Essential Findings in 15 Patients Autopsied within 12 Hours and 15 Patients Autopsied after more than 60 Hours Postmortem*

	Autopsy performed within 12 hours	Autopsy performed after more than 60 hours
Female	10	5
Male	5	10
Clinical myocardial infarct possible	1	[illegible]
Presumed heart death	6	7
Malignant disease	4	6
Suppurative infection	5	8
Cerebral attack	4	3
Embolus of the lung arteries	1	1
Other essential findings*	2	0
Severe oedema of lungs	5	[illegible]
Hearth weight (gm)		
less tran 300	3	3
300 to 449	9	10
450 and over	3	[illegible]
Circumscribed fibrosis of heart	3	[illegible]
Disperse fibrosis of heart	3	[illegible]
Atherosclerosis of coronary arteries		
Slight or no	8	11
Moderate	2	[illegible]
Severe	5	3
Thrombosis of coronary arteries	0	1

* In one case severe nec[illegible]ng pancreatitis and in another rupture of an aortic aneurysma

from one heart obtained from a 4 year old child, the average age of the patients was 70 years (46–85 years)

The most important clinico pathological data are shown in Table 1 In none of the cases was acute myocardial infarction suspected clinically or pathologically (conventional macroscopic examination of the myocardium) In all cases a complete autopsy was carried out The heart was sectioned transversely into ¾ cm slices from apex to the point of the papillary muscles of the left ventricle The remaining part was cut open and the myocardium flat cut Two myocardial slices corresponding to the base and the centre of the papillary muscles of the left ventricle were then incubated aerobically for 30 minutes in a solution consisting of substrate (sodium succinate and sodium lactate) and Nitro-BT in phosphate buffer at 37° C (1) In order to arrest the enzymatic reaction, the slices were transferred to Lillie's fluid and evaluated with regard to the colour intensity of the bluish black diformazan, its uniformity and the appearance, if any, of completely or partially unstained areas and their location Transmural sections of tissue were obtained from an intermediary myocardial slice, including the anterior and posterior walls and the septum These tissue sections were immediately frozen and, in most cases at once, but in a few cases not until 24 hours had elapsed, cut on a cryostat into 6 *mu slices. Dehydrogenase reaction* was then made as described above, without counter staining From the myocardial slices originally studied macroenzymatically, transmural tissue sections were also taken from the anterior and posterior walls and the septum, these sections were taken parallel and at right angles to the myocardial slices, in order to obtain, as far as possible, longitudinally cut muscle fibres involving all layers of the myocardium Similar to the involving of tissue cut on a cryostat these tissue sections were embedded in paraffin and, after fixation for 24 hours in formalin stained with haematoxylin eosin, van Gieson-Hansen and Gram stains

RESULTS

Out of the group examined within 12 hours (patient no 1–15), the macroenzymatic study revealed 13 hearts to be evenly and intensely blackish blue, and in the group examined later than 60 hours postmortem (patient no 16–30), 11 hearts presented a uniform and intense blackish blue stain disregarding distinctly fibrotic areas One heart (No 25) examined 60 hours postmortem, although uniformly blackish blue, was of a slightly reduced stain intensity Five hearts



Fig 1 Cross section of the heart corresponding to *the middle of the ventricles following macro-enzy*matic study In the subendocardial layers of the left ventricle, involving both papillary muscles, *nodular partly confluent areas are seen—interpret*ed as recent myocardial infarction This is in clear *contrast to the normal dark formazan* blue myocardium Note the ½ mm wide, non-infarcted formazanblue border most subendocardially in the otherwise infarcted area

presented smaller or greater unstained areas in the left ventricle In all of them an intense blackish blue zone could be seen about ½ mm subendocardially *in the unstained area* (*Fig* 1) and in all cases the remaining portions of the left and the entire myocardium of the right ventricle were intensely blackish blue

Patient No 4 76 year-old man Treated with prednison and endoxan for 9 years because of malignant lymphogranulomatosis His condition had gradually deteriorated He was admitted to hospital because of acute pain throughout the body and unmeasurable blood pressure. The EEG had changed markedly from previous recordings, ST depression having developed in leads V_{4-6} and isoelectric T waves in II III, aVR, aVF, aVL *He* died before serum enzyme determinations could be made

Autopsy was carried out 11½ hours postmortem Heart weight 420 g There was circumscribed fibrosis of the posterior wall of the left ventricle By macroenzymatic examination the subendocardial quarter of the ventricular wall, including both papillary muscles, presented *unstained nodules of* 4–6 mm, in some areas confluent The coronary vessels presented severe diffuse stenosing atherosclerosis without thromboses There was severe diffuse oedema of the lungs

Patient No 7 74 year-old woman with con

gestive heart failure and attacks of pulmonary oedema She had undergone three operations within the last month with lower limb amputations the last time 20 days before death Developed severe bedsores and died quietly No relevant ECG or serum enzyme examinations were available

Autopsy was carried out 12 hours postmortem *Heart weight 390 g* There was concentric hypertrophy of the left ventricle and circumscribed almost transmural fibrosis of the posterior wall of the left ventricle and subendocardial fibrosis Furthermore there were 3–4 weeks old infarction changes in the process of healing (*Mallory et al 1939*) in the subendocardial area of the septum and the posterior wall including the portion of the papillary muscle immediately adjacent to the septum By macro-enzymatic examination the proximal myocardial slice revealed unstained areas of 3–5 mm in the posterior wall of the left ventricle and in the remaining part of the posterior papillary muscle The coronary vessels presented severe stenosing atherosclerosis without thrombi and there was diffuse pulmonary oedema of slight to moderate severity

Patient No 23 59 year old man who had had bronchitis for many years Admitted with pneumonia During the last 36 hours before death the blood pressure was < 80 mm Hg ECG recorded 4 days before death was normal and no serum enzyme studies were carried out

Autopsy was performed 111 hours postmortem Heart weight 300 g The heart was flaccid pale and slightly yellowish in appearance Macro enzymatic study revealed a circular unstained area in the entire left ventricle mostly in the posterior wall where the unstained area was transmural whereas in the remaining part it was subendocardial only No atherosclerosis or thrombosis in the coronary vessels The lungs presented severe diffuse oedema and in addition severe purulent bronchopneumonia

Patient No 26 75 year old man who had had bronchitis for many years Admitted with cardiac arrest Heart beat was restored primarily but the patient remained in coma and further examination and treatment were therefore discontinued

Autopsy was carried out 101 hours postmortem *Heart weight 405 g* The posterior wall of the left ventricle presented circumscribed fibrosis Macro-enzymatic study revealed an unstained area shaped like a horseshoe in the subendocardial portion of the septum of the left ventricle and the adjacent parts of the anterior and posterior walls but also subtransmurally in a smaller part of the anterior wall The coronary vessels presented stenosing atherosclerosis and a fresh thrombus located 3 cm distally in the right coronary artery There was severe diffuse pulmonary oedema and purulent bronchopneumonia

Patient No 30 46 year-old woman operated on 9 days before death because of intracranial haematoma After the operation she was in coma and had brain stem attacks no relevant examinations were available

Autopsy was carried out 64 hours postmortem *Heart weight 360 g* The myocardium was pale and grey Macro-enzymatic study revealed an unstained area shaped like a horseshoe in the lateral portion of the left ventricle comprising both papillary muscles The injury penetrated nowhere deeper than into the subendocardial third of the wall There was sporadic atherosclerosis and no thrombosis in the coronary vessels There was severe diffuse pulmonary oedema and purulent bronchopneumonia

Evaluation of the microscopic dehydrogenase reaction was made blindly by both authors We ascertained the presence of totally unstained areas whereas because of the tissue sections particularly at the edges we did not attempt any quantitative evaluation of the strength of the enzyme reaction We discovered unstained areas corresponding completely to the findings made by macro enzymatic study, although lesser differences were observed in topography In the unstained areas, scattered enclaves of muscle fibres with preserved enzymatic activity were found In tissue sections from other hearts we found unstained areas of a few millimetres most often subendocardially These areas presented fatty infiltration or fibrosis of varying duration It was particularly difficult to evaluate areas with pigment containing macrophages newly developed capillaries collagen fibrils and scattered lymphocytes

The normal dehydrogenase activity was apparent in well preserved muscle fibres at magnification × 1000 as a slightly diffuse bluish counter staining and a strong bluish staining of the A bands of the myofibrils and furthermore as accentuated longitudinal striation of the muscle fibres most often of a diffuse bluish colour In a few areas this longitudinal striation appeared to be blue amorphous granules 1×2 mμ in size, of a linear structure and lying between the myofibrils Similar granules were also found corresponding to the poles of the sarcolemma nuclei

In all tissue sections isolated or confluent



F g 2 Dehyd ogenase react on (N tro-BT metl od) of long tud nally cut myocard al f bres Note the foc w th coarse organ zed and un form d formazan granules w th n o herw se normal myocard um (× 475)

foci were found in wh ch the accentuated transverse and longitudinal striation was re placed by coarse and more greenish blue diformazan granules (Fig 2) sl ghtly visible at magnification × 250 In many cases we found tl e d formazan granula of most un even size and shape and furthermore in some cases d sarranged (Fig 3) In the group of hearts examined before 12 hours post mortem we found in 3 of 15 cases uneven size and shape of granula and in only 1 of 15 cases d sarrangement of the granula In the group of hearts examined later than 60 hours postmortem 12 of 15 cases showed uneven size and shape and 7 furthermore d sarrange ment In 5 of the 7 cases with disarranged diformazan granules other organs presented purulent infect on In a few cases the spe cimens stained with Gram stain presented bacteria lying singly or in small accumula tions particularly in the epicardial vessels and their immediate surround ngs

By microscopic evaluat on of the haem atoxylineosin stained specimens attempts



F g 3 Dehydrogenase react on (N tro-BT method) of long tud nally cut myocard al f bres Note coarse nonun form and d sarranged d formazan granules (× 475)

were made to assess the incidence of the following parameters, qualitatively and quantitatively oedema, hyperaemia, appearance of *interstitial* neutrophilic granulocytes, nuclear changes (anisocaryosis, pyknosis, hyperchromasia, caryolysis and caryorrhexis) and changes in the stainability, fragmentation, cross-striation and any weakness of the muscle fibres Apart from the fact that no *granulocyte infiltration was found*, the other *parameters varied from one section to another* and even within the same section, apparently without any system However, the changes tended to be most pronounced in the areas which remained unstained during the dehydrogenase studies All things considered, the general impression of this examination was so uncertain that we abandoned quantitative analysis

DISCUSSION

Contrary to the series presented by *Nicolas et al* (1969) our series does not comprise normal individuals It is the material encountered during the daily work in an institute of pathology

The reason for our programming the macro-enzymatic examination with the addition of substrate and a reaction period of 30 minutes in all cases is our desire to develop a method which is easy to use, which does not give falsely positive results and which can be applied routinely We did not aim at developing a more sensitive method possibly with later addition of substrate and/or shorter and more flexible periods of incubation as that described by *Nachlas* & *Shnitka* (1963)

The 5 hearts which at macroscopic and microscopic enzymatic reaction did not, correspondingly, stain with formazan, because of reduction in or lack of dehydrogenase activity in these sections we evaluated as hearts with acute myocardial infarction

We have used this method for more than two years on over 900 hearts and the appearance and topography of the unstained areas found during the present study comply with our general experience In cases of myocardial infarction *it is characteristic that the three or four muscle fibres situated immediately below the endocardium are undamaged* (*Mallory et al* 1939, *Shnitka* & *Nachlas* 1963, *Seiferth* 1967, *Hort* 1968) Actually we found that *there was always a* blackish blue border *presenting diformazan* staining subendocardially in the otherwise unstained area (Fig 1) Similarly, the remaining part of the myocardium, and also the entire right ventricle, was normally and uniformly blackish blue During our daily use of the method we considered the *colour intensity* of the *narrow subendocardial* border and the lateral part of the myocardium of the right ventricle to be a built in control mechanism in each myocardial slice In our opinion, also the microscopic dehydrogenase study established the presence of infarction (*see below*) Also the case histories, the further pathoanatomical study of the heart and the coronary vessels and, in particular the fact that all *five patients* had diffuse pulmonary oedema—four of them of maximum severity —confirm our evaluation In a non selected consecutive autopsy series applying the macro enzymatic method, we have previously (*Andersen* & *Fischer Hansen* 1973) detected 24 per cent acute myocardial infarctions which cold not be revealed by other conventional macroscopic examinations and the incidence of *unexpected infarctions* in the present series is in absolute accord with this percentage

It was surprising to find complete similarity between *the findings at the macroscopic* and microscopic dehydrogenase reaction The rapid and unexpensive microscopic method can, therefore, be recommended in cases where conventional macroscopic evaluation of the myocardium *renders doubtful results* in respect to myocardial infarction or in cases where further details are desired Similar to *Knight* (1967) we find that also this method can be employed as late as 72 hours post mortem or even later, but in such cases we would recommend a check up on myocardial tissue obtained from the lateral part of the right ventricle Coarse formazan granulation

in particular at the margin of experimental infarctions, has been found by many investigators (*Shnitka & Nachlas* 1963, *Cox et al* 1968, *Kunde & Seidler* 1971), and these authors considered this to be a proof of reversible ischaemic myocardial changes In a survey published by *Jennings* (1969) concerning the early phase of myocardial ischaemia and infarction it appears, as should be expected, that ischaemic and autolytic myocardial changes present many common features, including the appearance of coarse formazan granules in the muscle fibres In our opinion, therefore, it is justifiable to consider the granulation and, first and foremost, its non uniformity and possible disarrangement as a sign of the degree of cadaverosis Hence, three of the hearts, in which we found it justified to consider the changes as being caused by recent myocardial infarction and not by autolysis, are among the least autolyzed hearts comprised by the present study (Nos 7, 23, and 30)

The results of the ordinary microscopical study were disappointing If a definite demarcation between the infarction of a duration of less than 12–24 hours and normal myocardium cannot be seen this method cannot be applied to human myocardium, as opposed to the conditions in non autolyzed experimental animals (*Jestädt & Sandritter* 1959 *Shnitka & Nachlas* 1963, *Fine et al* 1966) As regards weaviness of muscle fibres as an early morphologic sign of myocardial infarction, as described by *Bouchardy & Majno* (1972), we found this so frequently that in our opinion this criterion is of no importance However, it must be stated that these authors carried out autopsies within 12 hours postmortem and that the bodies were maintained at 4° C

Hence our conclusions are

1 In spite of autolysis, the macro enzymatic method is well suited for the detection of acute myocardial infarction in all cases up to and including the third postmortem day
2 Also within the same interval the micro-enzymatic method is well suited for the detection of infarction With increasing autolysis the formazan granules become coarse, uneven and disarranged
3 Conventional microscopic examination cannot be applied as the sole method in the detection of acute myocardial infarction

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Acta path microbiol scand Section A 82, 345–347, 1974

BRIEF REPORTS

PROLIFERATION OF HUMAN BONE MARROW IN NMRI NU/NU BOM MICE

Eivind B Thorling and Bent Pedersen

A method is described by which to grow human bone marrow under the kidney capsule of congenitally thymus aplastic, nude mice The technique is fairly simple, and since it may prove useful in experimental haematology our preliminary results are reported

The congenitally athymic nude mouse presents new possibilities for transplantation of allogeneic and even xenogeneic cells and organs A number of human tumours have been successfully transplanted to these mice and apparently, the structure and degree of differentiation is often well preserved (1, 3) Also serial transplantation has been successful in many instances with preservation of the histological characteristics of the primary tumour (2 5) These observations opened the possibility that human bone marrow cells might also proliferate and differentiate if transplanted to these mice The present report demonstrates proliferation by human bone marrow cells transplanted to the subcapsular space of the murine kidney The species specificity of the proliferating cells was verified by karyotype analysis

NMRI mice nu/nu Bom non inbred, were supplied by Dr *C H Friis* (Gl Bomholtgaard Ltd, DK 8680 Ry Denmark) The mice were bred and kept at SPF conditions by the breeder and transferred to our institute one week before use We kept the mice under conventional conditions in a separate room at 27° C The mice were handled by one technician only and no other persons were allowed access to the room Male mice were used exclusively The mice were fed ordinary laboratory chow (Rostock mixture) and water ad lib The bedding coarse saw dust was not sterilized

On the day preceding the transplantation the mice in groups of 5 were treated with 950 rads from a cobolt 60 source The irradiation was given to suppress the negative feedback on the donor marrow cells from the normal marrow components of the recipient After the irradiation, the mice were given Pentrexyl® (ampicillin) 2 5 mg/day intraperitoneally and were powdered all over the body with Topicin® (neomycin sulphate bacitracin 1 2) These measures appeared to be necessary and fairly effective in preventing gross infections of the mice in the granulopenic period following the irradiation If petechiae were pronounced, the Pentrexyl® injections were usually stopped on day 6–7 in order to prevent fatal bleeding

The human bone marrow was obtained by sternal puncture The patients (15 in all) presented a variety of different malignant disorders In most cases the marrow was considered normal or slightly hyperplastic but also marrow from patients with chronic myeloid leukaemia, acute lymphoblastic and acute myeloblastic leukaemia and myelomatosis was used in the experiments Twenty to 40 μl of the marrow cell suspension was injected per mouse After aspiration the marrow was added to 200 μl of medium TC 199 with 1/10 of a drop of heparin (Heparin Leo® 5,000 i u per ml) and kept cold on ice until use Care was taken not to disrupt the marrow particles The mixture was aspirated into a 1000 μl syringe through a coarse needle The syringe was then mounted with a finer needle and kept continuously cold on ice

The mice were anaesthesized by Nembutal (Abbott) 0 6 mg/10 g mouse and placed on their back on the operating board The hind limbs were extended backwards to either side whereas both forelimbs were fixed on the right side of the mouse This twist made the left kidney pop out rather easily after a small incision under the left curvature

The marrow suspension was injected on the convex side of the kidney under the capsule Before the skin was sutured, ample Topicin® powder was applied on the sutured peritoneum

Left alone the mice would now survive for approximately 10 days On sacrifice, dark red, grey spotted areas ranging between 2 5 mm in size, sometimes 1–1 5 mm thick, were seen on the kidney, often mantlelike encapsulating one pole of the

Received 20 xi 73 Accepted 11 i 74

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* Sponsored by the *Danish Cancer Society*

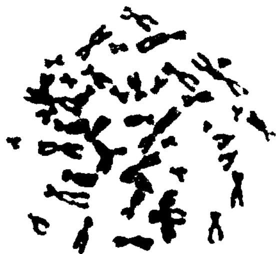


Fig 1 Human metaphase from a bone marrow graft from a patient with haemolytic anaemia



Fig 2 Localization of the graft under the kidney capsule



Fig 3 Haemopoietic area in a marrow graft under the kidney capsule

kidney The graft was excised Imprint preparations were stained with May Grunwald Giemsa stain In some experiments, the whole kidney was fixed and sectioned for histological examination For karyotyping the mice received 0 3 ml of a 0 04 per cent Colcemid (Ciba) solution 4 hours before they were killed The cells in the mantlelike graft were loosened by gentle teasing suspended in medium TC 199 and prepared for karyotype analysis according to the technique of *Tjio* & *Whang* (4) Metaphases were spread by air drying and stained with Giemsa s stain

On day 10 after transplantation generally very few cells were in mitosis whereas on day 6 7 relatively frequent cell divisions were observed Karyotyping was attempted in 6 cases and the presence of 2, 3, 3 and 3 human metaphases (Fig 1) was demonstrated in 4 cases haemolytic anaemia chronic myeloid leukaemia, myelomatosis and acute myeloid leukaemia Although human metaphases were demonstrated only in these cases the cytology of the imprint preparations and the histology of the sectioned grafts were compatible with the presence of human cells (Figs 2 and 3)

In the cases in which human metaphases were observed, murine metaphases were also seen It is not clear whether these cells represented regeneration of renal tissue, growing murine capillaries, fibroblasts or perhaps even haemopoietic cells of murine origin This last possibility, however, appears remote since endogenous haemopoietic colonies were extremely rare in the spleen of the animals after 950 rads

Aided by a grant from *Carl Schepler and Wife's Bequest, the Irma Foundation*

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Acta path microbiol scand Section A *82*, 347–348, 1974

SEARCH FOR TUMOUR RELATED BLOOD GROUP A AND B ACTIVITY IN ORAL SQUAMOUS CELL CARCINOMAS

E Dabelsteen

The blood group antigens A and B are not only located on the erythrocyte but occur in other mammalian cells and in secretions (8, 11) The fact that blood group active receptors disappear from human cells during development of malignancy and impending malignancy has been used as a diagnostic tool to detect malignant tumours (2, 6) It appears from some reports, however, that no decrease, rather an increase in blood group A reactivity occasionally may be seen in malignant tissue (3, 4 7), this applies to tumours of the oral, laryngeal cervical, and gastric mucosa It has been suggested that this positive reaction is caused by a tumour associated antigen cross reacting with blood group antigen A (4) This suggestion is supported by the finding of a tumour related antigen, the carcino-embryonic antigen, with blood group A like activity (10) In the present work, this problem has been further investigated by examining squamous cell carcinoma of the oral cavity of patients in blood group O for the presence of blood group A and B like substances. If a tumour antigen cross reacting with blood group substances A or B is produced in oral carcinomas it should be detected in these cases

Received 25 i 74 Accepted 25 i 74

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Material and methods

The material comprised surgical specimens from 22 squamous cell carcinomas of the oral cavity All patients were of blood group O The carcinomas were examined for the presence of A- and B-like substances using a double layer immunofluorescence staining technique with blood group antiserum and conjugate as described by *Dabelsteen* (1) The fluorescence microscope, filter systems, staining technique, and control reactions were identical to those described in previous papers (1, 5) To ensure maximum sensitivity of the staining reaction normal buccal mucosa from two non secretors (one blood group A and one blood group B) was included to serve as controls in the staining reactions. A activity as well as B-activity were known to be low in these cases (1)

Results and discussion

No blood group A or B-like substances were detected in the 22 carcinomas Both controls were reacting positively The lack of A- or B-activity in squamous cell carcinomas involving the oral cavity seems to indicate that these tumours do not generally produce a tumour antigen which is able

to cross react with blood group substances A or B These findings are in contrast to those obtained in studies of glycoprotein extracts from neoplastic gastric mucosa where 15 out of 24 cases demonstrated blood group specificity different from that of the hosts red cells (9) Similar results have been obtained by Hakkinen who found A like antigens in gastric tumour tissue of some patients of blood groups other than blood group A (7) The present findings are not in consistent with the concept that the blood group A activity in some oral carcinomas from group A persons is caused by a "real' A antigen produced by the cancer cells, the possibility that the A activity is related to a tumour antigen is less probable

The present work was supported by "*Daell Fonden'*, Copenhagen, Denmark and Grant DE 1358 from *The National Institute of Dental Research, National Institutes of Health*, Bethesda, Maryland, USA

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Acta path microbiol scand Section A 82, 349–357, 1974

INFLUENCE OF CYCLOPHOSPHAMIDE TREATMENT AND NEONATAL THYMECTOMY ON GLOMERULAR CHANGES IN RATS WITH LONG-TERM ALLOXAN DIABETES

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The effect of cyclophosphamide treatment and neonatal thymectomy on glomerular lesions in rats with long term alloxan diabetes was studied There were no significant differences between treated and untreated diabetic rats in the occurrence of glomerular IgG, graded and measured basement membrane thickness, and mesangial area In cyclophosphamide treated animals, however, there was a tendency toward reduced thickening of the glomerular basement membrane without decrease of glomerular IgG and of the degree of diabetes, as measured by diuresis The results do not support the assumption that immunological mechanisms are important in the pathogenesis of glomerular disease in diabetes

The occurrence of immunoglobulins in small vessel walls, including the glomerular capillaries, in diabetic subjects is well documented (*cf* 22) The underlying mechanisms of this protein accumulation are, however, essentially unknown, immunological reactions have been proposed by several investigators (*cf* 22) Furthermore, it is still unsettled whether the immunoglobulin deposition plays any role in the development of the microvascular disease in diabetes If immunological mechanisms are involved in the pathogenesis of diabetic microangiopathy it seems reasonable to anticipate that neonatal thymectomy and/or treatment with an immunosuppressive drug such as cyclophosphamide would affect the development of lesions of this kind In the present study this was investigated in rats with long term alloxan diabetes These animals develop glomerular lesions resembling the diffuse glomerulosclerosis in diabetes in man (4, 5), including deposition of immunoglobulin G (IgG) (6, 15) Studies of this kind have apparently not been performed previously

Received 12 x.73 Accepted 12 xii 73

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MATERIAL AND METHODS

Details concerning animal care, alloxan injection and diabetes control have been given in a preceding report (4) Albino rats of both sexes of highly inbred R strain were used They were maintained on commercial food pellets and water *ad libitum* Diabetes was induced at 3 months of age by an intravenous alloxan injection (55 mg/kg) under kidney protection The diabetic state was mainly controlled by monthly tests for glucosuria (Clini

TABLE 1 *Survival Rates of the 4 Main E... of the Number o...*

Sex	Untreated diabetic rats			Cyclophosph treated diabetic rats		
	No of living rats		Per cent survivors	No of living rats		Per cent survivors
	at 3½m	at 15m	at 15m	at 3½m	at 15m	at 15m
♂	128	21	16	87	11	13
♀	90	24	27	74	14	19

stix®, Ames Company) and measurements of 24-hour urine output adjusted to weight (4) Non-fasting blood glucose concentration and urine glucose content were usually determined 2 weeks and 6 and 12 months after diabetes induction All rats used in the present study had a permanent diabetes with a mean weighted diuresis of at least 20 ml/100 g/24 h, corresponding to a blood glucose level of at least 200 mg/100 ml (4) No insulin was given

Nondiabetic controls were either left untreated or they received injections of saline at 3 months of age (4) The untreated diabetic (120 rats) and nondiabetic (111 rats) animals referred to in this study are the same as those presented in preceding reports (4, 5, 6)

Cyclophosphamide administration From the third week and onwards after alloxan injection, 180 (97 males and 83 females) of the diabetic rats were treated every week with one intraperitoneal injection of cyclophosphamide (Sendoxan®)*, 7 5–10 mg/kg The drug was supplied in vials containing 100 mg of dry powder which was dissolved in sterile water and saline just before use The treated rats were weighed once a week The therapy was continued until the animals were sacrificed or died spontaneously Total peripheral blood leucocyte counts were carried out in some rats but there was no significant difference in cell number in cyclophosphamide treated (mean 24,500 cells/mm³) and untreated diabetic rats (mean 27 600 cells/mm³) Nine (5 males and 4 females) nondiabetic controls were also treated with weekly injections of cyclophosphamide, 10 mg/kg, from 4 to 15 months of age

Thymectomy thymus weights Seventy-two (37 males and 35 females) otherwise untreated diabetic rats had undergone neonatal thymectomy The operation was performed under hypothermia 72 hours after birth as described by *Janković et al* (8) The removed thymus tissue was not weighed completeness of thymectomy was checked *post mortem* by naked eye inspection Only animals with less than 50 mg of residual thymus parenchyma were included in this study The mean thymus weight in 15 month old nondiabetic control males was 285 mg ± 65 (SD) and in females 177 mg ± 49 (SD) In non thymectomized diabetic rats of the same age the weight was significantly lower ($p<0.001$), 133 mg ± 53 (SD) and 111 mg ± 36 (SD), respectively The lowest thymus weight recorded among these rats was 55 mg

Kidney biopsies At the age of 6, 9, 12 and 15 months, the rats in the experimental groups accounted for above were laparotomized in ether anaesthesia and kidney biopsy specimens for ordinary light microscopy were taken In most animals, the left kidney was also perfused with saline *in situ* for immunofluorescent studies (6) Tissue from the perfused kidney together with specimens for ordinary light microscopy were processed mainly according to *Sainte Marie* (19) Most of the rats were sacrificed afterwards, but about 20 per cent were allowed to live up to 15 months and were subjected to repeated laparotomies and kidney biopsy Kidney specimens for transmission electron microscopy were obtained from some 15 month-old rats

Cursory post mortem examinations were sometimes made on rats dying spontaneously in order to establish the cause of death of the animals In addition, more detailed autopsies on 6 rats were performed by veterinary pathologists at the State Veterinary Institute in Stockholm

Light microscopic investigations Staining of 5 µ thick sections was performed with the periodic acid Schiff (PAS) and van Gieson techniques (4) On coded PAS stained sections the average thickness of the PAS positive glomerular basement membrane of each specimen was graded semiquantitatively This was done on an arbitrary 4 point scale 1+ 2+, 3+ and 4+ (4) Specimens from most of the 15 month-old rats were also sectioned at 3µ and stained with PAS for quantitative determination of the mesangial regions by point counting method (4) The mesangial area was expressed in per cent of the total corpuscular area within Bowman's capsule

Immunofluorescent studies Sections of biopsy specimens from perfused kidneys were processed for immunofluorescent staining as described previously (6) An indirect technique was used The

* kindly supplied by AB Pharmacia Uppsala

l Groups of Rats at 15 Months of Age in per cent at 3½ Months of Age

Thymectomized diabetic rats			Untreated control rats		
No of living rats		Per cent survivors at 15m	No of living rats		Per cent survivors at 15m
at 3½m	at 15m		at 3½m	at 15m	
36	8	22	40	32	80
34	8	24	35	32	91

first layer consisted of an unconjugated rabbit serum against rat IgG or complement (β_{1C}) These sera were prepared in this laboratory (6) For the second step, a commercial fluorescein conjugated sheep anti rabbit immunoglobulin (Statens Bakteriologiska Laboratorium, Stockholm) was used Appropriate control stainings (6) were negative as regards specific fluorescence All stained sections were assessed blind in a Zeiss fluorescence microscope The intensity of glomerular fluorescence was graded semiquantitatively as 0 1+, 2+ or 3+

Electron microscopic measurements The details of the technique have been given in a preceding paper (5) Epon embedded kidney tissue from the following 4 groups of female rats was used *vi* 5 nondiabetic controls, 8 untreated diabetic animals 8 cyclophosphamide treated diabetic animals and 3 thymectomized diabetic animals Measurements of glomerular capillary basement membrane thickness were performed on electron micrographs (5) using 2 methods one described by *Jorgensen & Bentzon* (9) and the other by *Weibel & Knight* (21) The former method was applied to 3 glomeruli of each of the rats mentioned above Measurements according to *Weibel & Knight* were performed on 5 glomeruli from each of 5 controls from 5 untreated diabetic animals and from 5 cyclophosphamide treated diabetic animals

Statistical methods Student's t test was employed for testing differences between means Results of graded basement membrane thickness and of mesangial fluorescence for IgG were analysed using the *Wilcoxon* rank sum test Comparisons of survival rate between different groups of rats were performed with the chi square test $P < 0.05$ was chosen as the level of significance for all tests

RESULTS

In Table 1, the survival rate of the main experimental groups is shown At 15 months of age survival of the cyclophosphamide treated diabetic rats appeared to be somewhat reduced as compared with that of the untreated diabetic animals However, the difference was not significant Non specific pneumonia seemed to be the predominant cause of death in all groups Ketonuria and severe uraemia were never observed Therefore, the deaths did not appear to be attributable to ketoacidosis or uraemia

The mean body weights of the diabetic animals of corresponding sex in the various groups did not differ significantly at 4 months of age Untreated diabetic rats showed a tendency (for males significant at the 5 per cent level) to increasing body weight with age (Table 2) In contrast, the tendency of the cyclophosphamide treated diabetic animals, was the reverse The mean body weight of the 15 month old drug treated diabetic females was significantly ($p < 0.001$) lower than that in the group of untreated diabetic females

The mean of the weight adjusted urinary output (ml/100 g/24 h) of the untreated diabetic animals in the age groups 12 and 15 months did not differ significantly from the diuresis of the treated diabetic animals in the corresponding age and sex groups The values obtained in the groups of 15 month old animals are shown in Table 3

The results concerning glomerular basement membrane thickness relative mesangial area and mesangial IgG in the untreated controls and diabetics have been reported previously (4 5 6) The following Tables include the results obtained in the groups of untreated diabetic animals

Table 4 presents the results of the light microscopical semiquantitative grading of the basement membrane thickness in the *glomer-*

TABLE 2 *Body Weights (g) at 4 and 15 Months of Age in the 3 Main Experimental Groups of Allo xan Diabetic Rats Who Underwent Kidney Biopsy at the Age of 15 Months*

Sex	Untreated diabetic rats			Cyclophosph treated diabetic rats			Thymectomized diabetic r	
	Weight			Weight			Weight	
	at 4 months	at 15 months		at 4 months	at 15 months		at 4 months	at 15 months
	Mean ± SD	Mean ± SD	n	Mean ± SD	Mean ± SD	n	Mean ± SD	Mean ± SD
♂	209 ± 25	234 ± 45	18	216 ± 30	208 ± 30	11	214 ± 22	200 ± 41
♀	163 ± 22	177 ± 30	21	152 ± 16	137 ± 27	14	160 ± 10	163 ± 26

In a few rats, body weight was not recorded on both occasions and these animals are therefore not in cluded in the Table
SD = standard deviation, n = number of rats

TABLE 3 *Mean Weight-Adjusted Diuresis (ml/100g/24h) of the 3 Main Experimental Groups of 15 Month Old Alloxan Diabetic Rats*

Sex	Untreated diabetic rats		Cyclophosph treated diabetic rats		Thymectomized diabetic rats	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
♂	49 ± 12	21	53 ± 7	11	54 ± 15	8
♀	52 + 21	24	60 ± 17	14	51 ± 16	8

SD = standard deviation, n = number of rats

TABLE 4 *Glomerular Basement Membrane Thickness, Semiquantitatively Graded in the Light Microscope, in the 3 Main Experimental Groups of Alloxan Diabetic Rats at Various Ages*

Thickness	Age in months									
	4		6		9		12		15	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
	Untreated diabetic rats									
4 +									1	
3 +					1	1	5	2	17	20
2 +			4	3	8	5	13	3	3	4
1 +	8	8	4	4	2	3		1		
	Cyclophosphamide treated diabetic rats									
4 +										
3 +							1		8	8
2 +					3	4	2	1	2	4
1 +			2	3	1	1			1	2
	Thymectomized diabetic rats									
4 +									1	1
3 +							2		5	5
2 +					3	1		2		2
1 +										

TABLE 5 *Light Microscopic Determination of the Mean Relative Mesangial Area (Mesangial Area in per cent of Total Corpuscular Area within Bowman's Capsule) in the 3 Main Experimental Groups of 15 Month Old Alloxan Diabetic Rats*

Sex	Untreated diabetic rats		Cyclophosph treated diabetic rats		Thymectomized diabetic rats	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
♂	13 2 ± 1 7	14	13 1 ± 2 5	11	13 4 ± 2 0	6
♀	13 9 ± 1 9	24	14 1 ± 1 2	14	15 0 ± 1 7	8

SD = standard deviation, n = number of rats

uli Rats that had undergone kidney biopsy on several occasions are represented by the result from the last biopsy The results of serial biopsies from one and the same rat have been given in a preceding report (4) The 15 month-old cyclophosphamide treated diabetic rats, especially the females showed some tendency to less thickening as compared with the untreated diabetic animals of the same age The differences, however, were not significant Relative mesangial area (Table 5) did not show this tendency As regards mesangial regions, there were no significant differences between the untreated, cyclophosphamide treated, and thymectomized diabetic rats of corresponding sex at 15 months of age

Hyaline, intensely PAS-positive glomerular deposits resembling fibrinoid caps in human diabetic nephropathy, have been described previously in the untreated alloxan diabetic rats of both sexes (4) Such accumulations were seldom seen in the cyclophosphamide treated and thymectomized diabetic females Tubular and interstitial lesions that have been reported earlier (4) were of the same kind and roughly of equal frequency and severity in the treated and untreated diabetic groups of corresponding age and sex

Among the nondiabetic rats treated with cyclophosphamide, 3 animals of each sex sur

TABLE 6 *Semiquantitatively Assessed Specific Fluorescence for IgG in the Mesangium of the 3 Main Experimental Groups of Alloxan Diabetic Rats at Various Ages*

Amount of IgG	Age in months: 4 ♂	4 ♀	6 ♂	6 ♀	9 ♂	9 ♀	15 ♂	15 ♀
Untreated diabetic rats								
3+		3	2	3	1	1	1	5
2+	4	2	5	3	2	4	7	6
1+	2	3		1	3	1	2	4
0	2				1	1	3	1
Cyclophosphamide treated diabetic rats								
3+						1	1	4
2+					1	1	1	4
1+							2	
0							3	
Thymectomized diabetic rats								
3+							1	2
2+							3	3
1+							3	1
0							1	1

TABLE 2 *Body Weights (g) at 4 and 15 Months of Age in the 3 Main Experimental Groups of Alloxan Diabetic Rats Who Underwent Kidney Biopsy at the Age of 15 Months*

Sex	Untreated diabetic rats			Cyclophosph. treated diabetic rats			Thymectomized diabetic rats	
	Weight			Weight			Weight	
	at 4 months	at 15 months		at 4 months	at 15 months		at 4 months	at 15 months
	Mean ± SD	Mean ± SD	n	Mean ± SD	Mean ± SD	n	Mean ± SD	Mean ± SD
♂	209 ± 25	234 ± 45	18	216 ± 30	203 ± 30	11	214 ± 22	200 ± 41
♀	163 ± 29	177 ± 30	21	152 ± 16	137 ± 27	14	160 ± 10	163 ± 26

In a few rats, body weight was not recorded on both occasions and these animals are therefore not included in the Table
SD = standard deviation n = number of rats

TABLE 3 *Mean Weight Adjusted Diuresis (ml/100g/24h) of the 3 Main Experimental Groups of 15 Month-Old Alloxan Diabetic Rats*

Sex	Untreated diabetic rats		Cyclophosph. treated diabetic rats		Thymectomized diabetic rats	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
♂	49 ± 12	21	53 ± 7	11	54 ± 15	8
♀	52 ± 21	24	60 ± 17	14	51 ± 16	8

SD = standard deviation n = number of rats.

TABLE 4 *Glomerular Basement Membrane Thickness Semiquantitatively Graded in the Light Microscope in the 3 Main Experimental Groups of Alloxan Diabetic Rats at Various Ages*

Thickness	Age in months: 4		6		9		12		15	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Untreated diabetic rats										
4									1	
3+					1	1	5	[illegible]	17	20
2			4	3	8	5	13	3	3	4
1+	8	8	4	4	2	3		1		
Cyclophosphamide treated diabetic rats										
4+										
3+							1		8	8
2					3	4	2	1	2	4
1+			2	3	1	1			1	2
Thymectomized diabetic rats										
4+									1	1
3+							2		5	5
2+					3	1		2		2
1+										

TABLE 5 *Light Microscopic Determination of the Mean Relative Mesangial Area (Mesangial Area in per cent of Total Corpuscular Area within Bowman s Capsule) in the 3 Main Experimental Groups of 15 Month Old Alloxan Diabetic Rats*

Sex	Untreated diabetic rats		Cyclophosph treated diabetic rats		Thymectomized diabetic rats	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
♂	13 2 ± 1 7	14	13 1 ± 2 5	11	13 4 ± 2 0	6
♀	13 9 ± 1 9	24	14 1 ± 1 2	14	15 0 ± 1 7	8

SD = standard deviation n — number of rats

uli Rats that had undergone kidney biopsy on several occasions are represented by the result from the last biopsy The results of serial biopsies from one and the same rat have been given in a preceding report (4) The 15 month-old cyclophosphamide treated diabetic rats, especially the females showed some tendency to less thickening as compared with the untreated diabetic animals of the same age The differences, however, were not significant Relative mesangial area (Table 5) did not show this tendency As regards mesangial regions there were no significant differences between the untreated, cyclophosphamide treated and thymectomized diabetic rats of corresponding sex at 15 months of age

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Amount of IgG	Age in months 4 ♂	4 ♀	6 ♂	6 ♀	9 ♂	9 ♀	15 ♂	15 ♀
	Untreated diabetic rats							
3+		3	2	3	1	1	1	5
2+	4	2	5	3	2	4	7	6
1+	2	3		1	3	1	2	4
0	2				1	1	3	1
	Cyclophosphamide treated diabetic rats							
3+						1	1	4
2+					1	1	1	4
1+							2	
0							3	
	Thymectomized diabetic rats							
3+							1	2
2+							3	3
1+							3	1
0							1	1

TABLE 7 *Ultrastructurally Measured Mean Thickness (Å) of the Glomerular Basement Membrane of the 3 Main Experimental Groups of 15 Month Old Alloxan Diabetic Female Rats*

Measuring method	Untreated diabetic rats		Cyclophosph treated diabetic rats		Thymectomized diabetic rats	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
Jørgensen & Bentzon (9)	3210 ± 230 (3210 ± 230)	8	3060 ± 340 (2850 ± 260)	8	3220 ± 230	3
Weibel & Knight (21)	*3240 ± 170*	*5*	*3030 ± 270*	*5*		

Numbers within brackets are the results of measurements by the *Jørgensen & Bentzon* method on the same 5 rats as those measured by the *Weibel & Knight* method
SD = standard deviation, n = number of rats

vived for biopsy at 15 months of age The light microscopical picture of their kidneys *did not deviate from that of the untreated* control rats

Table 6 shows the results of the immuno fluorescence studies concerning glomerular IgG When IgG occurred in the glomeruli, it was regularly demonstrated in the mesan gium Sometimes a concomitant fluorescence was also seen along the capillary walls in a linear, interrupted pattern The incidence and amount of mesangial IgG did not differ significantly in the three groups of diabetic rats Complement (β_{1C}) could not be demon strated with certainty *in any glomerulus if the Sainte Marie* embedding method (19) was used However, as reported previously, β_{1C} was detected in the mesangium of some di abetic rats if frozen unfixed tissue was used (6)

The results of ultrastructurally measured glomerular basement membrane thickness in 15 month-old female alloxan diabetic rats have been presented in detail previously (5) *The number of diabetic rats investigated*, the mean thickness and standard deviation in each group are evident from Table 7 Some cyclophosphamide treated diabetic rats ex hibited a somewhat smaller thickness than the untreated diabetics but the difference be tween the two groups was not significant irrespective of whether the method of *Jørgen sen & Bentzon* (9) *or Weibel & Knight* (21) was *used Measurements were also performed* in 3 thymectomized diabetic females As re gards the mean thickness, no significant dif *ference between this group and the untreated* diabetic group could be demonstrated

DISCUSSION

Cyclophosphamide has been shown to be a powerful immunosuppressive agent *in the rat* impairing both humoral antibody responses and cell mediated immunity (*cf* 3) In the present study, cyclophosphamide treatment did not give any decrease of glomerular IgG in alloxan diabetic rats An explanation of this finding might be that the drug *doses* used were too low To inhibit certain experi mental autoimmune diseases of the thyroid and central nervous system in the rat, *cyclo*phosphamide was given at a minimum *dose* of 25–35 mg/kg per week (16 17) In these studies, the drug was administered *intraperi toneally daily or 5 days a week during 16–17* days At a dose level of 5–7 mg/kg per week little or no suppressive *effect on experimental allergic* encephalomyelitis was obtained (16) Therefore, the doses given in the present se ries, *7 5–10 mg/kg once a week* might have been inadequate *for immunosuppression* It must be pointed out, however that the drug *was administered* for several months in the present investigation A direct comparison of doses used in the various studies is *therefore* not possible Moreover in a pilot study where higher doses of cyclophosphamide were used

15 25 mg/kg per week the mortality was unacceptably high all treated diabetic rats died within 9 months

Tubular cell and glomerular lesions have been reported to occur in normal rats treated with cyclophosphamide (about 15 20 mg/kg per week i p) for up to half a year (10, 20) *Kallenbach & Schattenfroh* (10) described swollen capillary loops in the glomeruli and *Tessmann et al* (20) observed thickening of the glomerular basement membrane These observations were not compared with observations in untreated age matched controls The present study does not support the above observations

As to the relative mesangial area there were no significant differences between the groups of diabetic animals treated and not treated with cyclophosphamide Some decrease though not significant, of glomerular basement membrane thickness seemed to occur in the treated groups notably in the female group The degree of diabetes in treated and untreated animals as measured by diuresis, was comparable One explanation *of this tendency to reduction of thickness* might be a mechanism of selection, cyclophosphamide treated animals with more advanced glomerular changes would be more sensitive for the cytostatic treatment and die, while treated rats with less advanced glomerular lesions would survive However investigations of renal tissue from diabetic rats that were kept alive after the biopsy have not lent support to this hypothesis Some of these rats died spontaneously at varying intervals after the operation and some of them survived and were sacrificed at a later date The severity of the glomerular changes in those two groups did not differ significantly

Another possible explanation of the tendency to reduced thickening of the glomerular basement membrane in the cyclophosphamide treated diabetic rats is that the thickness of the basement membrane might be related to the body weight (*cf* 11) In fact, in the present study there was a tendency to a positive correlation between the graded glomerular basement membrane thickness and body weight in untreated diabetic animals at 15 months of age r_s (*Spearman's* rank correlation coefficient) for males = 0 37 (0 05<p<0 10), for females = 0 56 (p<0 01) A corresponding analysis of the cyclophosphamide treated diabetic rats did not reveal any correlation When the basement membrane thickness graded and measured, was adjusted to body weight (thickness per 100 g body weight), the tendency to differences of thickness in untreated and drug treated diabetic rats was abolished

Cell mediated immunity to glomerular basement membrane antigen has been demonstrated by migration inhibition tests in certain human renal diseases, such as glomerulonephritis and nephrotic syndrome, often with a concomitant deposition of immunoglobulins in the glomeruli (13, 14 18) It is not known however, whether cellular hypersensitivity participates in the development of the kidney lesions in these diseases Studies of cell mediated immunity in diabetic glomerulosclerosis do not seem to have been reported It was considered to be of interest to *see whether the cellular immune system* might contribute to the glomerular changes in alloxan diabetic rats It has been established that the adult rat thymectomized at birth has a general defect in delayed hypersensitivity reactions (1) In contrast the deficiency in humoral antibody responses is more variable, *e g* depending on the nature of the antigen given (2, 12)

In the present study neonatally thymectomized *alloxan diabetic rats did not differ* significantly from untreated diabetic rats concerning glomerular lesions including the occurrence of mesangial IgG *Jankovic et al* (8) considered thymectomy to be satisfactory if less than 100 mg residual thymus (for both sexes) was found in the rats at the end of experimentation Since some thymus atrophy occurs in untreated alloxan diabetic rats (7) as verified in the present investigation, the accepted upper *limit of residual thymus* weight in thymectomized diabetic animals was put at a lower level in this study, *ii* , 50 mg The majority of these rats had 0–10

mg residual thymus The lowest thymus weight recorded among the non-thymectomized diabetic animals was 55 mg It is therefore reasonable to assume that neonatal thymectomy performed in the present series resulted in impaired immunological functions The results mentioned above, concerning thymectomized rats, imply that cellular hypersensitivity does not play any essential part in the development of the glomerular lesions in alloxan diabetic rats

Thus, the present investigation has not given any support for the assumption that immunological mechanisms are involved in the pathogenesis of diabetic glomerulosclerosis

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This work was supported by grants from the *Medical Faculty, University of Umeå*, the *Swedish Diabetes Association* and the *Nordic Insulin Fund*

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MCG101-AA, A NEW ASCITES TUMOUR IN C_{57} MICE

1 *Induction Procedures and Some Cytological and Physicochemical Characteristics*

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A new ascites tumour, MCG101 AA in syngeneic C_{57}Bl/6J mice is described in relation to its solid progenitor, MCG101 SS The tumours show important differences in karyotype lectin agglutinability and thromboplasticity On the other hand, the tumours have similar transplantabilities, cross react immunologically and the solid ascites tumour, MCG101 AS has an electrophoretic mobility similar to that of MCG101 SS The usefulness of new ascites tumours for metastasis research is discussed

Ascites conversion of solid tumours represents a means by which to obtain highly dispersed tumour cell suspensions for experimental purposes Most ascites tumours in use have been serially transplanted for long, however, often in homologous host and represent extremes in tumour progression

In our laboratory attempts are made to produce ascites forms of several types of freshly induced tumours, primarily for use in metastasis studies The present report describes the conversion of a MC induced sarcoma into ascites form and some characteristics of the new tumour

Original Solid Tumour—MCG101-SS

This tumour arose in an inbred female C Bl/6J mouse given 1 mg 3 methylcholanthrene subcutaneously The antigenicity of the tumour and its metastasizability in early transplant generations and in immunologically altered hosts have been described by *Boeryd & Suurkula* 1973

It was originally a moderately differentiated fibrosarcoma (Fig 1 which has grown somewhat less differentiated (Fig 2) in the course of serial subcutaneous transplantation in the same inbred strain of mice in which it arose It has been preserved by frozen storage in several generations It was the only one among five newly induced tumours which transformed to ascites form within 10 transplantation generations by the procedure to be described

Ascites Transformation

The procedure adopted was similar to that described by *Mellgren et al* (1966) including testicular and intraperitoneal transplantations The procedure is shown schematically in Fig 3 Here it is also shown that transplantation of MCG101 SS with tumour mince or as a monocellular enzymatically produced suspension only produces solid tumour growth intraperitoneally On the other hand the solid tumour to arise when AA cells are transplanted s c (MCG101 AS see below!) gives rise to ascites tumour when retransplanted i p, even after 6 s c transfers The AA and AS forms even though infiltrating in the peritoneal wall, show a much lesser degree of infiltration than the SS form (cf *Ringertz et al* 1957) Hence, the criteria for an irreversible conversion to ascites form (*Klein* 1955 a b) are fulfilled

Cytology and Growth Characteristics of MCG101 AA

The morphology in smears has hitherto been fairly constant The tumour grows intraperitone

Received 21 ix 73 Accepted 13 xii 73

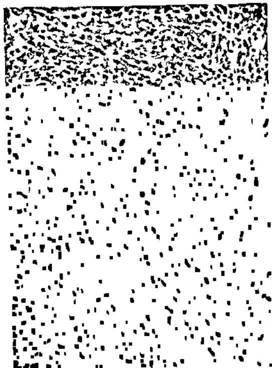


Fig 1 MCG101 SS in transfer generation 5 Low power view showing the whirled structure with fus form, fibroblast like cells (Htx eosin)

The tumour cells have a marked cellular and nuclear pleomorphism (Fig 4 and 5) Their irregular nuclei often contain one or more distinct nucleoli There is a frequent occurrence of multinucleated giant cells ($\simeq$ 5 per cent of the population) The mitotic index varies with the age of the tumour, and this parameter is being reported together with other population kinetic data obtained after DNA labelling procedures (*Hagmar* & *Lundin* 1973)

Some preliminary *cytochemical* studies have been performed with the tumour Except in direct smears the cells were stained after being washed twice in Hank's BSS (dilution 1 10 from ascites, agitation and centrifugation at 80 g for 10 min) After the washing processes, $\simeq 10^5$ cells in 1 ml were put in a small glass "chimney" glued onto a glass slide and allowed to settle for 1 hour They were fixed in a wet state, usually according to *Mowry* & *Millican* (1953) in absolute ethyl alcohol at –10° C As control served mast cell suspensions from rats and sections of cartilage, colon, small bowel, and salivary glands from mice (*Spicer* & *Henson* 1967)

The cells are negative in Mowry & Millican's PAS procedure, but the ascites fluid in direct smears is faintly positive In stainings for acid

ally in a highly viscous ascites In Papanicolaou or Giemsa stained smears the ascites fluid forms a thready material enmeshing the tumour cells This sometimes gives the impression of aggregates (Fig 4) but in less dense areas the cells are well dissociated The same is true if the cells are diluted and washed in aqueous media by which procedure practically monocellular suspensions can be obtained (*Hagmar* 1974 a) In such procedures the addition of DNase (ICN Nutritional Biochemicals Cleveland, Ohio, USA) in concentrations up to 100 μg/ml helps in dissociating the cells after centrifugation but it does not dissolve the original slimy material which is also Feulgen negative and hence not, at least not predominantly, DNA derived (cf *Moscona* 1962)

The admixture of inflammatory cells is always slight occasional leucocytes and peritoneal macrophages are present but they do not constitute more than maximally 10 per cent of the cell number Erythrocytes, however, may be present in great numbers and after day 3 after transplantation, the ascites often is grossly haemorrhagic But sometimes an ascites practically free of blood is obtained as late as day 10 after routine transfer The reasons for this variability in blood contamination, which causes experimental difficulties, is as yet unknown



Fig 2 MCG101 SS in transfer generation 46 Note the preserved structure of a fibrosarcoma, although poorly differentiated (Htx-eosin)

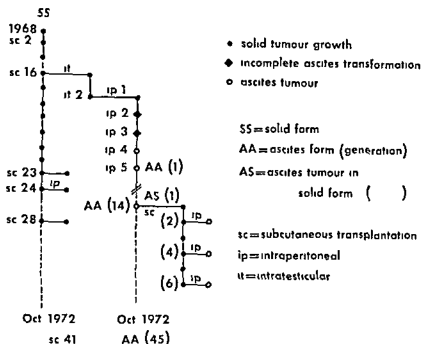


Fig 3 Transplantation procedures used to transform MCG101 SS into ascites (AA) form Transplantation tests with SS and AS (solid ascites) i p show that SS only gives solid tumour growth when transplanted i p while AA transferred for 6 generations s c (AS form) immediately reverts to ascites form i p

mucopolysaccharides with toluidine blue at pH 4 and 0 5 (*Spicer* 1962), the cells show only orthochromatic stainability and mainly of nuclei, also Hale s colloidal iron method (*Mowry* 1958) stains mainly nuclei and shows no distinct affinity for the cell periphery Neither did an aldehyde fuchsin—Alcian blue procedure (*Spicer* & *Meyer* 1960) produce any evidence of a stainable 'glycocalyx', as described for other tumours (*Gasic* & *Baydak* 1962)

The Solid Ascites Tumour (MCG101 AS)

If transplanted subcutaneously MCG101 AA *cells give rise to solid tumours, called the AS form* according to *Klein* (1955 a b) Histologically these tumours are quite undifferentiated without the *spindle cells and whirled structure that are present* in corresponding SS generations (Fig 6) If trans planted s c with small solid pieces (~ 3×1 mm³) by trochar it grows faster if anything than the SS form reaching 1 cm s diameter in 10–12 days It can be brought into a well dissociated, viable sus pension (> 90 per cent by the Trypan blue test) by the same trypsin DNase procedure that produces good suspensions also from 101 SS and other solid tumours (*Nortby et al* 1966 *Knutson et al* 1971)

The AS cells in suspension mentioned in the comparisons below were obtained in this way and as a final medium they were suspended in Parker 199 medium (P), with or without syngeneic serum (S, 5 or 10 per cent e g P_{9} S_{5} or P_{90} S_{10}), de pending on their use The AA/AS tumours were from the 3rd–10th transplantation generations and the SS tumour correspondingly in its 21st 30th generation

Comparisons between the SS and/or AA AS Tumours

Transplantability

Detailed studies of transplantation characteristics in syngeneic animals will be given elsewhere (*Hag mar* 1974 a, b) But the minimal doses giving takes *in 4/4 animals* subcutaneously (s c) intraperitone ally (i p) and 6/6 animals intravenously (i v) determined from serial dilutions in P_{0} S_{5} were

	s c	i p	i v
SS form	10^4	10^6*	10^5
AA "	10^5	10^2	10^4
AS "	10^5	10^2	10^4

* 3/4 from 10^3 and 10^2 cells

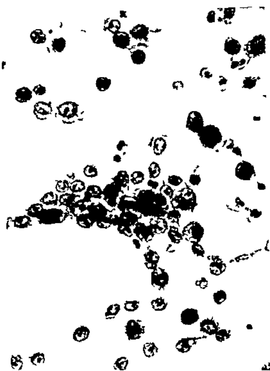


Fig 4 MCG101 AA in transfer generation 10 to show large cell aggregate in slimy material Such aggregates are dissolved in aqueous media (Papanicolaou)



Fig 5 MCG101 AA with multinuclear giant cells and blood contamination (Papanicolaou)

In *allogeneic animals* (inbred A/Sn and C_3H mice) SS and AA cells (in P medium) only gave rise to slight, transient growth from the two highest cell doses used (5×10^6, and 10^6 cells in 4 animals per dose and strain) Thus at these dose levels none of the two tumour forms was allotransplantable

Antigenicity

A cross reactivity test was performed with SS and AA cells As immunization, 5×10^6 X irradiated cells (10 000 R) in P medium were given s c. Fifteen days thereafter the same number of irradiated cells was given as a booster dose and at the same time a challenge dose of viable cells was given (cf *Revész* 1960) The challenge doses and the incidences of resulting s c tumours are shown in Table 1 When tumours arose, the growth was always progressive and mortal for the hosts

As seen in the table, both tumour forms are antigenic in syngeneic hosts and in immunized animals the takes frequency is reduced by one or more dose levels The SS and AA forms also cross-

TABLE 1 … *Mice* … *and Incider* … *Dose* … *of* 5×10^6 … *Dose*

Type of tumour for immunization for challenge	AA AA	(controls) – AA	AA SS	SS AA	SS SS	(controls) – SS
Challenge dose						
10 cells	4/4	–	4/4	1/4	4/4	–
10^5 „	2/4	4/4	4/4	2/4	1/4	4/4
10^4 „	0/4	4/4	0/4	0/4	1/4	4/4
10^3 „	0/4	0/4	0/4	0/4	1/4	2/4

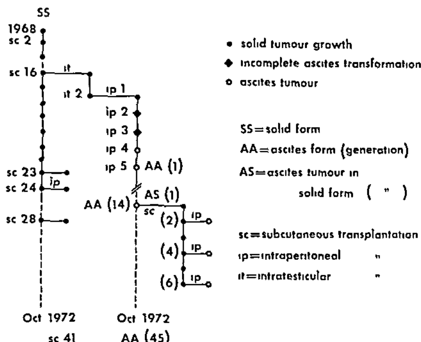


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The AS cells in suspension mentioned in the comparisons below were obtained in this way and as a final medium they were suspended in Parker 199 medium (P), with or without syngeneic serum (S, 5 or 10 per cent e g P_{95} S_5 or P_{90} S_{10}) depending on their use The AA AS tumours were from the 3rd 10th transplantation generations and the SS tumour correspondingly in its 21st 30th generation

Comparisons between the SS and/or AA AS Tumours

Transplantability

Detailed studies of transplantation characteristics in *syngeneic animals* will be given elsewhere (*Hagmar* 1974 a b) But the minimal doses giving takes in 4/4 animals subcutaneously s c intraperitoneally (i p) and 6/6 animals intravenously (i v) were determined from serial dilutions in P_{95} S_5 were

	s c	i p	i v
SS form	10^4	10^4*	10^6
AA "	10^3	10	10^6
AS "	10^3	10	10^4

* 3/4 from 10^1 and 10^2 cells

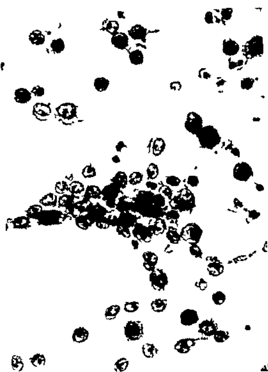


Fig 4 MCG101 AA in transfer generation 10 to show large cell aggregate in slimy material Such aggregates are dissolved in aqueous media (Papanicolaou)

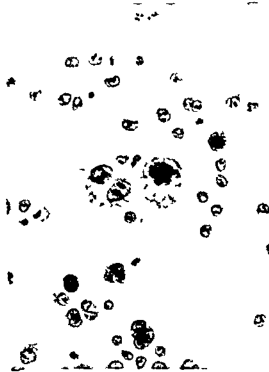


Fig 5 MCG101 AA with multinuclear giant cells and blood contamination (Papanicolaou)

In *allogeneic animals* (inbred A/Sn and C_3H mice) SS and AA cells (in P medium) only gave rise to slight, transient growth from the two highest cell doses used (5×10^6 and 10^6 cells in 4 animals per dose and strain) Thus at these dose levels none of the two tumour forms was allotransplantable

Antigenicity

A cross reactivity test was performed with SS and AA cells As immunization 5×10^6 X irradiated cells (10 000 R) in P medium were given s c Fifteen days thereafter the same number of irradiated cells was given as a booster dose and at the same time a challenge dose of viable cells was given (cf *Revész* 1960) The challenge doses and the incidences of resulting s c tumours are shown in Table 1 When tumours arose the growth was always progressive and mortal for the hosts

As seen in the table both tumour forms are antigenic in syngeneic hosts and in immunized animals the takes frequency is reduced by one or more dose levels The SS and AA forms also cross

TABLE 1 *Immunization—Challenge Scheme for MCG101 SS and AA in Syngeneic C_5 Bl/6J Mice and Incidence of s c Tumours after Challenge with Graded Cell Doses Immunization and Booster Dose of 5×10^6 X Irradiated Cells Given s c 15 Days before and Simultaneously with the Challenge Dose*

Type of tumour		(controls)				(controls)
for immunization	AA	–	AA	SS	SS	–
for challenge	AA	AA	SS	AA	SS	SS
Challenge dose						
10^6 cells	4/4	–	4/4	1/4	4/4	–
10^5	2/4	4/4	4/4	2/4	1/4	4/4
10^4	0/4	4/4	0 4	0/4	1/4	4/4
10^3	0/4	0/4	0/4	0/4	1/4	2/4

react as evidenced by the mutual protection they offer in immunized hosts The results for 101 SS agree with those obtained by *Boeryd & Suurkula* (1973), although they used a solid piece of viable tumour for immunization and achieved an even greater protection of immunized animals. These authors also tested and verified the specificity of the response by immunizing and challenging with another solid MC sarcoma (MCG 102) which did not cross react with 101 SS

Karyotypes

Chromosome analyses were performed after Colcemid (Ciba) treatment of tumour bearing animals, 3 doses of 4 μg/g b w being given i p in 0 2 ml at intervals of 1 5 hours The squash—orcein method described by *Hsu & Klatt* (1958) was used on washed ascites cells or enzymatic suspensions from the SS tumour Photographs were taken with oil immersion objective (100 ×) The counts include only metaphases in which preserved cellular outlines exclude the loss of chromosomes in the preparation

The studies comprise as yet only data from a few generations In Fig 7, the idiograms of the SS and AA forms in roughly corresponding generations are given As can be seen, there is a change of modality from 41 chromosomes in the SS form to 42 in the AA form There was otherwise a similar scatter of hyperdiploid (> 40 chromosomes) metaphases in the two tumour All chromosomes were

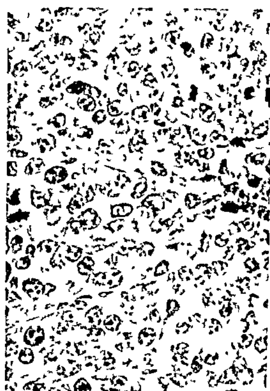


Fig 6 MCG101 AS from AA transfer generation 4 The tumour is undifferentiated and rather monomorphous (Htx eosin)

TABLE 2 *Lectin Agglutinability of MCG101 Cells*

Tumour form	Con A μg/ml Wga	Concentrations of agglutinins 400 1 8	200 1 16	100 1 32	50 1 64	25 1 128	0 0
AA	Con A	+	+	0	0	0	0
	+ MG	0	0	0	0	0	0
	Wga	+ +	+	0	0	0	0
	+ NAGA	0	0	0	0	0	0
AS	Con A	+ + +	+ + +	+ +	+ +		0
	+ MG	0	0	0	0	0	0
	Wga	+ + +	+ + +	+ +	+ +		0
	+ NAGA	0	0	0	0	0	0
SS	Con A	+	0	0	0	0	0
	+ MG	0	0	0	0	0	0
	Wga	+ + + +	+ + +	+ + +	+ +	0	0
	„ + NAGA	0	0	0	0	0	0

Con A = Concanavallin A MG = α methyl D glucopyranosid Wga = wheat germ agglutinin from inactivated wheat germ lipase NAGA = N acetyl D glucosamine

AA 2 × washed from ascites

AS trypsinized suspension from solid tumours

SS „ „

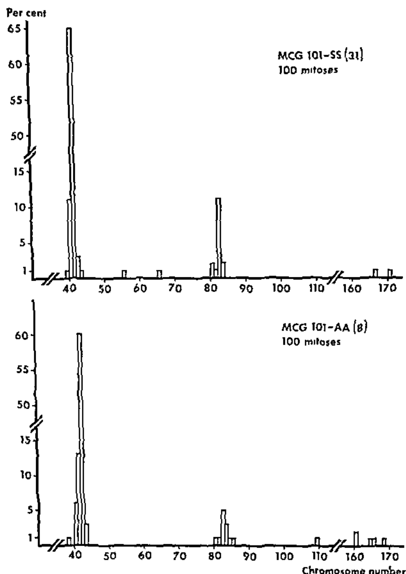


Fig 7 Idiograms of MCG101 SS and AA chromosome number *Note the difference in stemline number*, 41 in SS and 42 in AA The two hyperdiploid tumours have a similar, small scatter of other aneuploid metaphases

telocentric and neither tumour showed any marker chromosomes The occurrence of minutes was occasionally noted in both tumours

Lectin Agglutinability

The aggregation in the presence of two types of lectins Concanavallin A (Con A) and wheat germ agglutinin (Wga) was tested as described by *Kapeller & Doljanski* (1972) The lectins (Con A from ICN Nutritional Biochemicals Wga prepared from wheat germ lipase /Sigma/ according to *Burger & Goldberg* 1967) were diluted to isotonicity with distilled water and saline before being used for serial dilutions with Eagle s MEM in the aggregation test The tests were performed at room temperature and read after gentle agitation for 30 minutes The degree of aggregation was scored 0–+ + + + + and the tests were performed blindly with and without the presence of the respective sugar inhibitor in 0 2 M concentration (Con A α methyl D glucoside, Wga N acetyl D glucosamine obtained from Sigma Chemical Company, S t Louis, USA) The concentrations of the agglu-

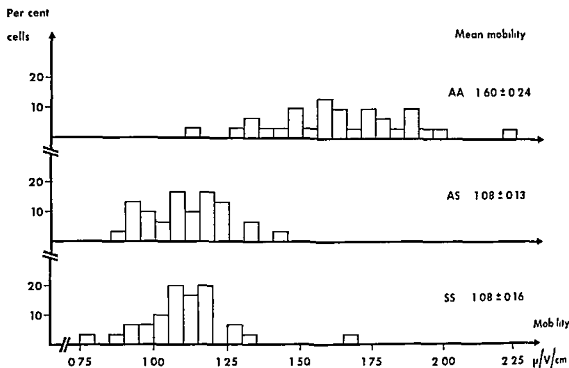


Fig 8 Histogram of MCG101 cell electrophoretic mobilities Thirty cells were measured in both directions *Note the greater scatter among AA cells and the similarity in mean mobilities of AS and SS cells*

tinins and the outcome of the test are shown in Table 2

The AS tumour was specifically agglutinated by both lectins The SS form was almost unsensitive to Con A, but highly reactive with Wga On the other hand the AA form was only weakly agglutinated by the highest concentrations of the two lectins

Electrophoretic Mobility

As a measure of the net negative surface charge, the mobility of tumour cells was determined in a cylindrical cell apparatus (*Bangham et al* 1958) The procedures were those used previously (*Hagmar & Norrby* 1973) with a voltage gradient of 50 V between the grey sintered platinum electrodes, and using Parker 199 as suspension medium. The ascites cells were washed twice in Parker medium before use The SS and AS cells were washed once after the dissociation procedure (*Norrby et al* 1966)

The mobilities are given in Fig 8 AA cells were more heterogenous than the trypsinized cell types, but had also a much higher mobility and hence a greater net surface charge density The difference between these and the AS and SS cells, which had similar mobilities, was about 28 per cent

Thromboplasticity

The thromboplastic activity was determined as described before (*Hagmar & Boeryd* 1969), from the clotting activity of frozen and thawed tissue homogenates in serial dilution on a standard rat plasma. The coagulation times inversely proportional to the thromboplastic activity applying to the SS and AS tumours and to some normal tissues are shown in Fig 9

MCG101 SS showed a weak activity only slightly higher than that of muscle The AS tumour had a distinctly greater activity comparable to that of lung tissue Brain homogenate had a higher activity than any tumour as previously described (*Hagmar & Boeryd* 1969)

COMMENTS

As pointed out by others (e g *G Klein* 1951 b), ascites tumours may be superior to solid tumours in many experimental situations In our metastasis studies (*Surveys Boeryd 1966, Hagmar* 1970 *Hagmar & Norrby* 1973) it has frequently been difficult to obtain acceptable suspensions from solid tumours Of course, if the main objective is

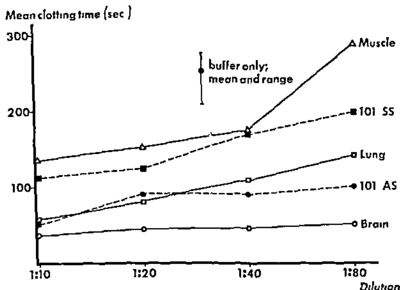


Fig 9 Mean clotting times in seconds for standard rat plasma in serial dilution with tissue homogenate in buffer Thromboplastic activity inversely proportional to clotting times

to relate cell surface characteristics to meta stasis patterns, as we now try to do, the re peatedly obtainable, well dissociated suspen sions of cells undamaged by mechanical trauma or enzymes, as in ascites tumours, are very useful

One of the difficulties to be encountered when solid tumours are to be converted into ascites tumours is the resistance of many tumours towards transformation This problem has been studied in detail by *G Klein* (1951 a b) and *E Klein* (1955 a, b) Although new procedures, as the testicular transplantation introduced by *Mellgren & co workers* (1966), may be helpful, it probably has to be accepted that ascites adapted mutants cannot always be obtained

Another difficulty, once the conversion has succeeded, is to obtain an adequate characterization of the tumour in relation to the tumour of origin and other ascites tumours Indeed, in addition to new environmental conditions s p , any number of genetic changes may take place in the transformed tumour and/or in the tumour of origin Among the factors which hitherto have attracted much attention are chromosomal changes (*Hauschka & Levan* 1953, *Hauschka et al* 1971), light and electron microscopic morphology (*Bergstrand & Ringertz* 1960, *Boeryd et al* 1968) and infiltrative behaviour (*Ringertz et al* 1957)

Chromosomally no new aspects emerged, but as has been the case with other tumours (*Mellgren et al* 1966, *Hauschka et al* 1971), the karyotype will provide a tool by which further genetic alterations may be visualized

The infiltrative behaviour of the tumour will be further discussed in conjunction with metastasis studies performed with the SS and the AS forms of MCG101 (*Hagmar* 1974 b) The two forms have different metastasis pat terns when transplanted subcutaneously and it is our hypothesis that this circumstance, like differences in metastasizibility (cf *Klein* 1955 b, *Ringertz et al* 1957), may be related to differences in surface properties of the various tumour forms So far a characterization of the surface properties has just com menced Ultrastructural aspects, as revealed by transmission and scanning electron microscopy, will be separately dealt with (*Hagmar et al* 1973) It was not possible to produce well dissociated and viable mechanical suspensions from 101 SS and AS to test whether the electro negative surface charge would in-

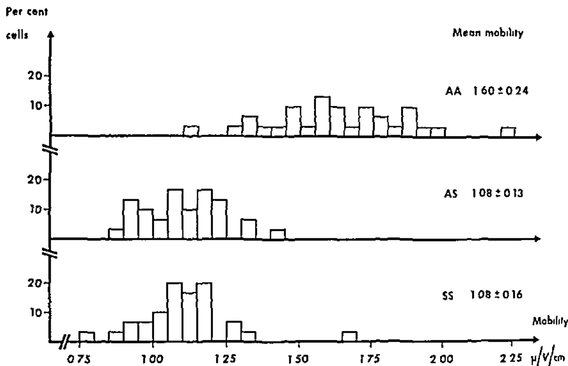


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MCG101-AA, A NEW ASCITES TUMOUR IN C_{57} MICE

2. *Protocol of* in vivo *Transplantation Studies in Comparison with the Solid (SS) and Solid Ascites (AS) Tumours*

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The transplantability of a newly induced ascites tumour, MCG101 AA, was compared with that of the solid tumour of origin, MCG101 SS, and the ascites tumour in solid form, MCG101 AS. The AA and AS tumours required lower cell inocula for growth i.p. than s.c., while SS had a similar transplantability at these two sites. If injected i.v., all tumours gave rise to extensive lung tumours. In addition, the SS tumour formed numerous extrapulmonary tumour takes, more than AS and AA, the latter giving rise only to a few such tumours. The evaluation of the results is complicated by different survival times after injection of the three tumours. But it is suggested that the dissemination pattern was altered by ascites transformation of the tumour. Possible mechanisms involved, such as cell surface alterations, are discussed.

The transformation of a solid tumour into ascites form is a clonal selection phenomenon, as demonstrated by *Klein* (1955). The criterion of selection is the ability of the cells to thrive and divide in a free state intraperitoneally. This probably is associated with alterations of the surface properties of the cells (*Klein* 1951, 1955, [illegible] 1959 [illegible]

the host. It is, however, very incompletely tested how the ascites transformation affects the *in vivo* behaviour of the cells. This is true also of the metastatic dissemination pattern, which may be related to surface properties of tumour cells (*Gasic & Baydak* 1962, *Hagmar* 1970, 1972 a).

It was the aim of the present study to test the transplantability of a newly induced ascites tumour, MCG101 AA, in comparison with the solid tumour of origin, MCG101 SS, and the solid ascites tumour, MCG101 AS. Critical take doses were determined for the subcutaneous (s.c.) and intraperitoneal (i.p.) sites. The amount and distribution of tumours ('experimental metastases') in animals injected intravenously (i.v.) with different cell doses of the three tumours were recorded.

Received 1.x.73 Accepted 1.x.73

MATERIAL AND METHODS

Tumours. The tumour of origin is a solid MC sarcoma, MCG101 SS, from which an ascites tumour, MCG101 AA, was produced in transfer generation 16–23 as described earlier (*Hagmar* 1973 a). The solid form of the ascites tumour, MCG101 AS, was produced by injecting AA cells (10^6) s.c. All tumours were carried in inbred syngeneic C_{57}Bl 6/J mice and all experiments were performed in such mice.

Suspension Procedures

MCG101 AA is growing intraperitoneally as a practically monocellular suspension in a characteristically slimy ascites (*Hagmar* 1974 a) To avoid aggregation, the ascites was diluted in Hank's BSS 1 10 immediately upon removal from the abdomen After thorough agitation, the cells were spun down (80 g, +4° C for 10 min), resuspended in the same volume of Hank's and re-centrifuged The cell pellet was resuspended in Parker 199 medium to which DNase (NBC, Cleveland, Ohio) was added in a concentration of 60 µg/ml and syngeneic serum to 10 per cent ($Parker_{90}$ $serum_{10}$ or P_{90} S_{10}) After haemacytometer counts of cell number and viability (Trypan blue) the suspension was diluted to the appropriate cell number with P_{95} S_5

MCG101-SS and -AS were taken on day 12–14 after transplantation when the tumours measured about 1 cm in diameter The suspensions were produced by a combination of mechanical procedures and trypsinization in a modified version of the procedure described by *Madden* & *Burk* (1961) The modifications (*Norrby et al* 1966 *Knutson et al* 1971) include DNase exposure after the final centrifugation and the addition of syngeneic serum at resuspension In the transplantation experiments to be described, P_{90} S_{10} was used at resuspension, while P_{95} S_5 was used to dilute the suspensions, similarly as for AA

At injection, one sample containing 5 × 10^5 cells was taken from each type of tumour cell suspension and filtered onto a Millipore filter with pore size 5 0 ± 1 2 µM for Papanicolaou staining and microscopic examination of numbers and sizes of cell aggregates (*Hagmar* & *Norrby* 1972) In study B, the average cell sizes were calculated from the filters by measuring the diameters of 10^3 randomly chosen cells using a Zeiss eye piece micrometer

Transplantation Experiments

Two studies (A and B) were performed Study A was a pre test of dose levels comprising only MCG101 SS and AA, taken in their 28th and 21st transfer generation respectively Study B includes SS (generation 28) and AA (generation 5), but also AS, produced from AA (gen 4) and transferred s c for 3 additional generations

Cell suspensions in serial dilutions were injected s c and i p in 4 animals per site and cell dose level The same suspensions were given i v in 4 animals per group in study A and in 6 animals per group in study B The relation between sexes was equal in all groups The injections were given in sequence, alternating between the injection sites from agitated suspensions stored on ice

The animals were observed daily for 4 months As regards those injected s c, the time of appearance of a palpable tumour was registered The time of spontaneous death of animals with i p and i v transplants was noted and these animals were autopsied as soon as possible, including registration of the gross tumour extent The lungs and livers were prepared for histological examination from several serial sections according to *Boeryd* (1966) Sections were also taken from each kidney and from any doubtful tumour In study B the lungs were weighed after formalin fixation as described before (*Hagmar* & *Boeryd* 1969)

TABLE 1 *Study A, MCG101-SS Cells Injected Subcutaneously (S c), Intraperitoneally (I p), and Intravenously (I v) in Graded Cell Doses*

Cell dose	S c	I p		I v				
	Incidence of takes	Incidence of takes	Mean survival time in days	Incidence of takes	Mean survival time in days	Incidence of lung tumours	Extra pulmonary tumours Incidence (number) and sites	
10^6	4/4	4/4	13	4/4	23	4/4	2/4§ (18)	Subcutis 7, fat 6 muscle 3 mesentery 1, adrenal 1,
10^5	3/4	4/4	19	4/4	27	4/4	3/4 (4)	Subcutis 2, muscle 1, heart 1
10^4	1/4	4/4	29	0/4	–	–	–	–
10^3	2/4	3/4*	35	0/4	–	–	–	–
10^2	0/4	0/4		0/4	–	–	–	–

* One of the takes occurred subcutaneously
§ Two spontaneously dead animals disappeared before autopsy

TABLE 2 *Study A, MCG101 AA Cells Injected s c, i p and i v in Graded Cell Doses*

Cell dose	S c Incidence of takes	I p Incidence of takes	I p Mean survival time in days	I v Incidence of takes	I v Mean survival time in days	I v Incidence of lung tumours	I v Extra pulmonary tumours Incidence (number) and sites
10^6	4/4	4/4	19	4/4	22	4/4	1/4 (1) fat 1
10^5	2/4	4/4	33	4/4	25	4/4	0/4
10^4	0/4	3/4*	31	4/4	36	4/4	0/4
10^3	0/4	2/4*	50	2/4	37	2/4	0/4
10^2	0/4	1/4*	–	0/4	–	–	–

* One of the takes occurred subcutaneously

TABLE 3 *Study B, Mean Cell Surface and the Relation between Single Cells and Cell Aggregates per 1000 Particles*

Type of suspension	Mean cell surface on filter (mm^2 10^{-6})	Number of single cells	Number of aggregates containing the following cell number 2	3	4	5	6
SS	43	868	109	16	7	1	
AA	66	845	109	31	10	4	1
AS	53	909	76	12	2	1	

RESULTS

Study A

The viabilities by dye exclusion was 89 and 90 per cent, respectively, in the SS and AA suspensions Unfortunately the Millipore filters were technically unsatisfactory and hence, it was not possible to assess the agregability and the cell sizes

In vivo (Table 1 and 2) there were no significant differences (X^2-test) in the capability of the two tumours to grow s c or i p, although SS had occasional s c takes at lower cell dose levels than AA AA was somewhat more prone to grow i p than s c From i v injected cells AA gave tumour takes at lower dose levels than SS in fact with similar cell doses as those needed for growth i p While SS killed the i p transplanted animals somewhat earlier than AA, the survival times of animals injected i v with either of the tumours were similar On the other hand, a most striking finding in i v injected animals was the occurrence of extrapulmonary SS tumours in contrast to AA Alas, 2 animals from the highest dose level were lost, thereby obscuring a little this difference (Table 1) No tumours were detected microscopically in liver or kidneys

Study B

By dye exclusion test, the SS, AA and AS suspensions had similar viabilities (88, 85 and 90 per cent, respectively) The proportion of single cells and aggregates and the mean cell surface on filter are shown in Table 3 As is evident, all suspensions were well dissociated and consisted of single cells and small cell aggregates —*It should be noted* that a maximal value of aggregation is obtained by this method, since cells may be layered in juxtaposition at the filtration The AA cells had the greatest cell surface, differing significantly from the AS cells by Student s t-test The

TABLE 4 *Study B, MCG101-SS Cells Injected s c , i p and i v in Graded Cell Doses*

Cell dose	S c	I p	I v				
	Incidence of takes	Incidence of takes	Incidence of takes	Incidence of lung tumours	Mean lung weight (± S D)	Extrapulmonary tumours Incidence (number) and sites	
10^6	4/4	4/4	6/6	6/6	0 35 (±0 06)	6/6 (66)	Subcutis 13, muscle 28, heart 2, fat 7, mesentery 3, kidney 7, lymph nodes 2, ovary 4,
10^5	4/4	4/4	6/6	6/6	0 65 (±0 28)	6/6 (25)	Subcutis 4, muscle 7, fat 3, mesentery 10, adrenal 1,
10^4	4/4	4/4	6/6	6/6	0 55 (±0 41)	2/6 (2)	muscle 1, adrenal 1,
10^3	2/4	3/4	4/6	4/6	0 63 (±0 28)	1/6 (3)	muscle 1, kidney 2
10^2	1/4	3/4	–	–	–	–	

– = dose level not used i v

TABLE 5 *Study B, MCG101-AA Cells Injected s c , i p and i v in Graded Cell Doses*

Cell dose	S c	I p	I v				
	Incidence of takes	Incidence of takes	Incidence of takes	Incidence of lung tumours	Mean lung weight (± S D)	Extrapulmonary tumours Incidence (number) and sites	
10^6	4/4	4/4	6/6	6/6	0 54 (±0 14)	0/6 (0)	
10^5	4/4	4/4	3/3*	3/3	0 34 (±0 07)	1/6 (2)	heart 2,
10^4	1/4	4/4	6/6	6/6	0 69 (±0 34)	2/6 (2)	mediastinum 1,
10^3	1/4	4/4	5/6	5/6	0 41 (±0 14)		mesentery 1,
10^2	0/4	4/4	–	–	–	2/6 (3)	muscle 1, mesentery 2

* 3 animals accidentally uninjected
– = dose level not used i v

TABLE 6 *Study B, MCG101 AS Cells Injected s c , i p and i v in Graded Cell Doses*

Cell dose	S c	I p	I v				
	Incidence of takes	Incidence of takes	Incidence of takes	Incidence of lung tumours	Mean lung weight (± S D)	Extrapulmonary tumours Incidence (number) and sites	
10^6	4/4	4/4	6/6	6/6	0 39 (±0 18)	3/6 (4)	Subcutis 1, fat 1, heart 2
10^5	4/4	4/4	6/6	6/6	0 30 (±0 08)	4/6 (7)	Subcutis 1 muscle 2, heart 2, mediastinum 1, adrenal 1,
10^4	0/4	4/4	6/6	6/6	0 27 (±0 10)	4/6 (4)	lymph node 1, muscle 3,
10^3	1/4*	[illegible]/4	3/6	3/6	0 35 (±0 20)	2/6 (3)	lymph node 1, adrenal 2
10^2	0/4	4/4		–	–		

* take i p
– = dose level not used i v

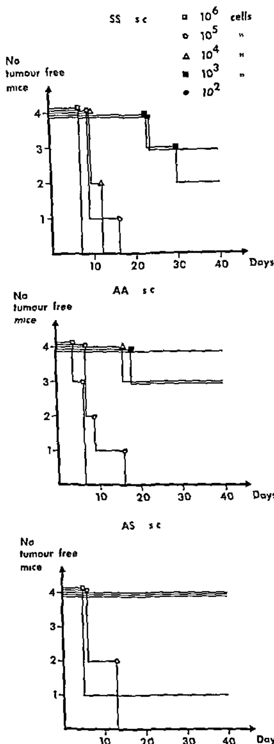


Fig 1 Study B s c takes frequency for SS AA and AS suspensions The time of appearance of a palpable tumour is indicated

AS cells in turn were larger than the SS cells

The results of the *in vivo* studies are given in Table 4–6 In addition, the incidence of takes, latency periods for s c tumours and survival times of animals injected i p and i v are given in Fig 1–3

The tumours appeared more virulent than in study A, i e both SS and AA took with smaller cell inocula at all sites, although the differences are not always significant The trend of AA in study A, namely growth of smaller cell inocula i p than s c, was now still more prominent The AS tumour shared this preference for the i p site, while SS grew almost as well subcutaneously Alas, the lowest cell dose (10^2) was not used i v in this study and hence, strict comparison with this injection compartment is not possible In some animals, however, all tumours grew *from doses as small as 10^3 cells, given i v*

The latency period for s c tumour development was similar for the three tumour forms (Fig 1) On the other hand, spontaneous death from i p and i v transplanted cells (Fig 2 and 3) ensued soonest in animals injected with AA cells, while AS killed the hosts faster than SS cells

All positive animals injected i v developed lung tumours and, as a rule, the lung tumours were the cause of death The average lung weights given in the tables reflect the amount of tumour at spontaneous death (cf *Hagmar* & *Boeryd* 1969) But since the survival times were different, the lung weights cannot be used for statistical comparison between groups The SS lung metastases were generally large, solid tumours while AA and AS lung tumours were small and scattered Animals given any of the ascites variants had, as a rule, also a slimy, haemorrhagic pleural exudate, which probably helped in killing the hosts sooner than those receiving SS cells

As in study A, the pattern of extrapulmonary tumours ("metastases") were characteristically dissimilar for the SS and AA forms While the SS tumour gave rise to numerous extrapulmonary takes only a few tumours were registered in AA animals The AS tumour fell in between in this respect No

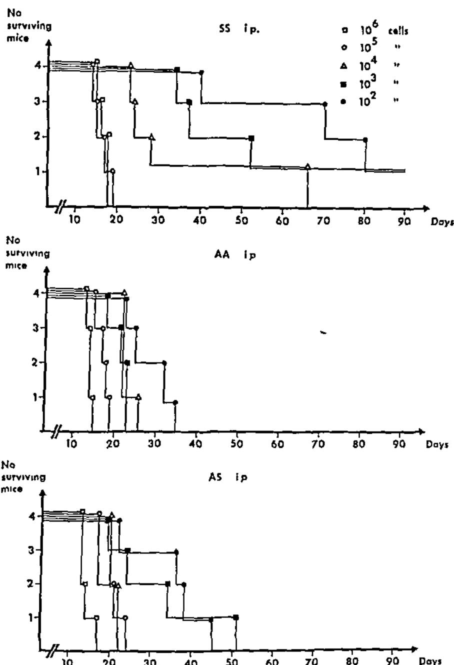


Fig 2 Study B, i p takes frequency for SS, AA and AS suspensions The time of spontaneous death is indicated

tumours were found histologically in livers, except in 3 mice given 10^6 SS cells

COMMENTS

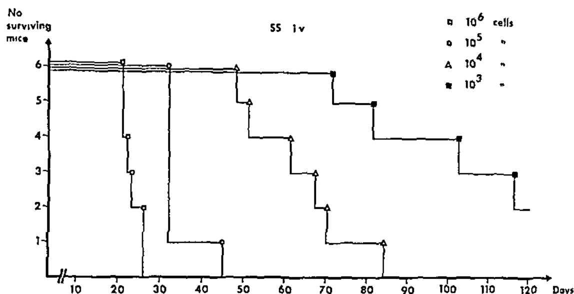
In the present experiments, a pre-test of cell doses, Study A, was performed with the solid (SS) sarcoma in its 28th generation and the ascites (AA) form in its 21st generation The test was repeated (Study B) with generations 28 and 5, respectively, which correspond in age, since the first AA generation corresponds to the solid generation 23 (*Hagmar* 1974 a) This was possible by frozen storage of the tumours

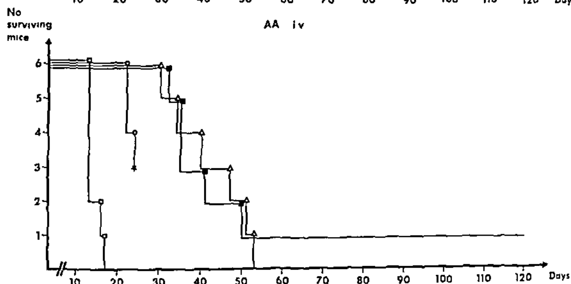
In these tests, the two forms of the MCG101 tumour seemed to possess a similar "virulence", judged by their overall takes frequencies *in vivo*. The fact that the critical dose levels for takes were lower in Study B than in A is difficult to explain by altered malignancy, since the SS tumour was in the same transfer generation and the AA tumour only some 15 generations apart. Other possibilities are that the actual tumours used after frozen storage and thawing were of a better quality in Study B—not reflected in the dye exclusion test—or that errors in sampling, counting and dilution procedures may be of this order. Anyhow, the AS tumour in Study B was similar to AA in transplantability and in preferring the i.p. to the s.c. transplantation site. This is very reasonable in view of the genetically determined irreversible character of the ascites transformation process (*Klein* 1955). Apparently, the trypsinization of AS did nothing to alter the transplantation characteristics s.c. and i.p., even though tumour development i.p. was somewhat quicker for the AA form.

If injected i.v., the 3 tumours would behave differently as revealed by the present method of recording the tumour extent in animals dying spontaneously. While SS, AA and AS all produced lung tumours, SS alone gave rise to widely disseminated metastases. AS gave distinctly fewer extrapulmonary takes in fewer animals. As regards AA, a still smaller number of extrapulmonary takes developed and preferentially in animals given small cell inocula. This fact, along with the shorter survival times of the AA animals, might indicate that the animals given large AA cells doses died before cells disseminated beyond the lungs had time to grow into visible tumours. This should be cautiously borne in mind when it is stated that the MCG101 tumour changed its dissemination pattern by the ascites transformation. That this may be the case is still a distinct possibility however and in fact, is true for the spontaneous spread of the 101 SS and AS tumours (*Hagmar* 1973 b). In Study A, where the survival times were comparable the difference in extrapulmonary tumours was also evident.

If the AA form is less prone to develop tumours outside the lungs, i.e. the first capillary bed encountered by the injected cells, a number of reasons for this have to be envisaged. The surface properties of the tumours, as they are now beginning to be characterized (*Hagmar* 1974 a), may play a role, as they undoubtly do in preserving the ascites form of growth. The adhesiveness of AA cells towards pulmonary endothelium may for instance be greater, although it is difficult to envisage how (cf. *Ryd et al.* 1973). The AA form has a higher negative cell surface charge density than AS and SS cells (*Hagmar* 1974 a) which presumably would prevent close contact with endothelium. This will be the case, however, only if the repulsion barrier is not overcome by cell protrusions such as ascites tumour cells may produce (*Boeryd et al.* 1968). Another possibility is that the tumour cells may be "glued" onto the endothelium by extraneous material, such as thrombocytes (*Jones et al.* 1971) or fibrin. The greater thromboplastic activity in AA AS compared to SS (*Hagmar* 1974 a) thus may cause fibrin precipitation in lung vessels and thereby a greater cell retention. This is possible in the artificial injection situation, where damaged cells and some cell debris are injected along with viable cells (cf. *Hagmar* 1972 b). This factor is much less likely to play a role in spontaneous spread where, as a matter of fact, the AS tumour does not show a preferential retention in the lungs (*Hagmar* 1974 b).

The mechanisms suggested do not explain why AS gave rise to a higher number of extrapulmonary tumours than AA. Again, however, the surface properties must be taken into consideration. Indeed, AS was subjected to the same dispersion procedures, including trypsinization, as SS. As shown previously (*Hagmar* & *Norrby* 1973), trypsinization may cause this type of alteration in tumour metastasis patterns. The mechanism is unknown, but the cell size might be one of the factors to determine where and when the


No surviving mice
SS iv
10^6 cells
10^5 "
10^4 "
10^3 "
Days


No surviving mice
AA iv
Days

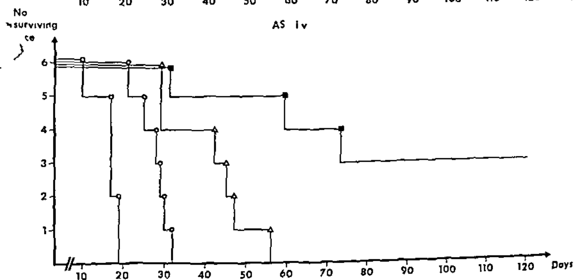

No surviving mice
AS iv
Days

Fig 3 Study B, i v takes frequency for SS, AA and AS suspensions The time of spontaneous death is indicated

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tumour cells will disappear from the blood The present method of measuring the cells on a Millipore filter was chosen because the AA tumour contains some non tumourous cells (*Hagmar* 1973 a) which can be excluded by this method Also the method, unlike other sizing procedures (e g *Hagmar* &

filtered out to a greater extent in lung capillaries But since even cell aggregates seem to pass lung vessels (*Lawrence et al* 1953, *Hagmar* & *Norrby* 1973), this probably is not a major mechanism Neither should differences in aggregability play any significant role in the present experiments where all suspensions were similarly well dissociated The cell size and aggregability are, however, factors which should be checked in metastasis experiments until their possible roles are better defined Also, the interpretation of the results will be more precise when the initial distribution of tumour cells can be followed in detail (cf *Fidler* 1970)

To sum up, the present results show that the ascites (AA) form of MCG101 has other dissemination characteristics than the solid tumour of origin (SS) Part of this difference may be due to suspension procedures, as revealed by comparison with the AS form Further studies are required to elucidate the precise mechanisms involved But the cloning of tumours, as by ascites transformation, may be a useful tool in studies of tumour dissemination

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The skilful technical assistance of Miss *Marianne Bremner* and Mrs *Lena Sandahl* are gratefully acknowledged

The study was supported by research grants from the *Swedish Cancer Society* (no 465 B72-03X)

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MCG101-AA, A NEW ASCITES TUMOUR IN C_{57}MICE

3 *Studies of the Spontaneous Metastasis Formation from the Resectable Solid Ascites Tumour in Comparison with the Solid Tumour of Origin*

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The metastasizing capacity of the solid ascites (AS) form of a new ascites tumour, MCG101 AA, was tested and compared with that of the solid sarcoma, MCG101 SS, from which the ascites tumour was produced The tumours, in roughly corresponding transfer genera tion, were transplanted subcutaneously into a hind leg of syngeneic animals Radical resection was performed by amputation of the leg at different intervals of time after transplantation The tumours started to metastasize after a similar time interval and produced meta stases in almost 100 per cent of animals carrying the tumours for 3 weeks Yet, the AS metastases killed the hosts sooner than the SS metastases The pattern of spread of the two tumours was also characteristically dissimilar SS metastases arose preferentially in the lungs, the AS metastases at other sites such as lymph nodes, heart or muscle

Little is known about the factors determining the metastasizing capacity of malignant tumours It has long been suspected, however, that the surface properties of tumour cells make them less "adhesive" to the point of origin—and thereby more easily detached—than normal cells On the other hand, they may adhere to the endothelium and thus grow in distant organs (*Coman* 1953, 1961) Further study has shown that surface properties may also determine tumour dissemination patterns, i e sites to which the cells migrate and grow into metastases (*Hagmar* 1970, 1972, *Hagmar* & *Norrby* 1972)

Comparison of the metastasizing capacity of an ascites tumour and that of its solid progenitor showed that the number of cells which moved from the ascites tumour to distant organs was higher than that of cells moving from its progenitor (*Klein* 1955, *Ringertz et al* 1957) This greater metastasizing capacity has been ascribed to cell surface changes, specifically to the higher electronegative charge density on the ascites cells (*Purdom et al* 1958) Yet, we do not know whether these findings apply to other systems as well

Our current studies of the newly induced ascites tumour, MCG101-AA, pay special attention to its surface properties In the present study, we investigated also its spontaneous metastasizing capacity in syngeneic animals, as compared with roughly corresponding generations of the tumour of origin, MCG101-SS

Received 1 x 73 Accepted 1.x 73

MATERIAL AND METHODS

Tumours

The ascites tumour, MCG101 AA was produced in inbred C_6 Bl/6J mice and originated from the solid MC sarcoma, MCG101 SS, in its 16th–23rd transfer generations, as previously described (*Hag mar* 1974a) The ascites tumour grows intraperi

toneally (i p) as a practically monocellular suspension If injected subcutaneously (s c), it gives rise to a solid undifferentiated tumour which is called MCG101-AS according to *Klein* (1955) It can be serially transplanted for several generations, but will revert to ascites (AA) form if transplanted i p

Transplantation and Resection Techniques

In the present studies the tumours were transplanted in syngeneic animals by sterilized trochars containing ∽ 3 × 1 mm³ solid tumour tissue The trochars were inserted s c on the lateral side of the right hind leg, close to the foot The tumour transplant was deposited near the knee and the wound was closed by nippers

The tumours were removed by amputation of the whole hind leg The skin was opened widely around the right hip and the thigh musculature was ligated at the highest possible sites The musculature and femur could then be cut by a pair of scissors just distal to the ligature This type of amputation, performed in ether narcosis caused very little bleeding and the animals recovered quickly It seemed to be a method more atraumatic than other, similar methods (*Ketcham et al* 1959, *Rudenstam* 1968) The right inguinal lymph node could also be easily identified and, if desired, removed

Registration of Tumours and Metastases

The tumour bearing leg was weighed after amputation and fixed in formalin From each tumour a piece was cut longitudinally along the leg, embracing also some of the musculature All animals, whether dead spontaneously or killed, were submitted to a complete autopsy including registration of gross tumours The lungs livers and any doubtful tumours and lymph nodes, were fixed in formalin on removal The lungs were weighed in a fixed state as previously described (*Hagmar & Boeryd* 1969), before being processed for histological examination together with the other material The type of serial sectioning described by *Boeryd* (1966) was used for lungs and livers

Experimental Design

Two consecutive studies A and B, were performed The ratio between sexes was equal in all groups

In *Study A*, 50 animals were transplanted as described above with each tumour The SS tumour was in its 19th transfer generation and the AS tumour was derived from AA generation 4 Ten animals were amputated on day 7 and 10 additional animals on day 14 after transplantation (=1 and 2 weeks) The remaining 30 animals were divided into two groups Animals in both groups were amputated on day 21 after transplantation (3 weeks) One of these subgroups was subjected to homolateral inguinal lymphadenectomy (*vide supra*) In the other subgroup, the lymph nodes were left intact

When animals in a group started to die spontaneously, all the remaining animals in that group would be killed In this way, the survival times given in Tables 1 and 2 were obtained

In *Study B*, performed with SS generation 24 and AS again from AA generation 4 the 1 week groups were omitted Ten animals with each tu

TABLE 1 *MCG101-SS, Study A, Spontaneous Metastasis Formation from Tumours Transplanted s c in the Leg and Subsequently Resected In Group IV, Homolateral Lymphadenectomy Was Performed at Amputation Survival Times of Animals in Groups I and II 91 Days in Groups III and IV 44 Days*

[G]roups	[In]terval between [tr]ansplantation [a]nd amputation	Mean leg tumour weight in grams (±S D)	Incidence of lung metastases	Mean lung weight in grams (±S D)	Extrapulmonary metastases Incidence	Extrapulmonary metastases Number	Extrapulmonary metastases Sites
[I]	1 week	1 08(±0 17)	1/10	0 28(±0 06)	0/10	0	
[I]I	2 weeks	2 29(±0 89)	2/10	0 28(±0 03)	0/10	0	
[I]II	3 weeks	3 71(±0 86)	13/14	0 29(±0 08)	4/14*	7	lymph node muscle kidney mediastinu[m]
[I]V	3 weeks + inguinal lymphadenectomy	3 74(±0 91)	15/15	0 34(±0 13)	1/15	3	muscle kidney mediast[i]n[um]

[*] One animal died accidentallly at [a]mputation

Groups interval between transplantation and amputation	Mean leg tumour weight in grams (±S D)	Incidence of lung metastases	Mean lung weight in grams (±S D)	Extrapulmonary metastases Incidence	Number	Sites
1 week	1 72(±0 25)	0/10	0 26(±0 01)	0/10	0	—
2 weeks	3 64(±0 76)	1/10	0 26(±0 07)	5/10	8	lymph nodes 6 subcutis 2,
3 weeks	*5 62(±1 84)*	*6/13 **	*0 32(±0 06)*	*11/13*	*46*	lymph nodes 29 heart 5 muscle 9 subcutis 1, adrenal gland 1, mediastinum 1,
3 weeks + lymph adenectomy	5 14(±1 56)	2/12†	0 28(±0 05)	9/12	19	lymph nodes 6 heart 3 muscle 10,

*Two out of the original 15 mice died before amputation
†Three out of the original 15 mice died before amputation

TABLE 3 *MCG101-SS, Study B Spontaneous Metastasis Formation from Tumours Transplanted s c in the Leg and Subsequently Resected The Animals Were Observed until Spontaneous Death*

Groups interval between transplantation and amputation	Mean leg tumour weight in grams (±S D)	Incidence of spont death (+ mean survival time)	Lungs Incidence of tumours	Lungs Mean lung weight in grams (±S D)	Extrapulmonary metastases Incidence	Number	Sites
2 weeks	2 03(±0 41)	3/10(72)	3/10	1 04(±0 65)	0/10	0	—
3 weeks	2 99(±0 82)	12/20(79)	12/20	1 12(±0 36)	3/20	3	lymph nodes 1, heart 1, fat 1,

mour were amputated after 2 weeks and 20 animals after 3 weeks A homolateral inguinal lymphadenectomy was performed on all animals The animals were observed for spontaneous death The observation time covered 6 months after which all remaining animals were killed and autopsied

RESULTS

The tumour and metastasis parameters in Study A are given in Tables 1 and 2 and those in Study B in Tables 3 and 4

Study A The continuous growth of the primary tumours in legs is reflected in successively higher tumour weights In all groups the AS tumours weighed significantly more ($p < 0.01$ by Student s t test) than corresponding SS tumours The lung weights were similar in all groups reflecting the fact that lung metastases, if present, generally were few In most cases, the SS *metastases* were grossly visible, however, while the AS metastases were microscopic and scattered in the parencyma

TABLE 4 *MCG101 AS, Study B, Spontaneous Metastasis Formation from Tumours Transplanted s c in the Leg and Subsequently Resected The Animals Were Observed until Spontaneous Death*

Group Interval between transplantation and amputation	Mean leg tumour weight in grams (±S D)	Incidence of spont death (+ mean survival time)	Lungs		Extrapulmonary metastases		
			Incidence of tumours	Mean lung weight in grams (±S D)	Incidence	Number	Sites
I 2 weeks	2 75(±0 88)	4/10 (43 days)	2/10	0 27(±0 02)	4/10	11	lymph nodes 4 heart 6, med astinum 1
II 3 weeks	5 31(±1 71)	14/16 (41 days)	5/16*	0 32(±0 09)	14/16*	64	lymph nodes 23 heart 30, muscle 9, med a stinum 2,

*4 animals among the original 20 died spontaneously prior to or at resection

Histological examination of the primary tumours confirmed s c growth in all cases Both SS and AS tumours infiltrated the underlying muscle and also the overlying skin No overt differences were detected in this respect, other than the different histology of the two tumours (*Hagmar* 1974 a)

The incidence of metastases rose with the duration of the primary tumours in both systems, but the tumour patterns were quite dissimilar While SS metastases were mainly located in the lungs, detectable AS metastases in this organ were present in, at most, 6 out of 13 mice (Group III, Table 2 On the contrary, in extrapulmonary organs where SS metastases were rare, AS metastases were frequently found, often many in one and the same animal

Homolateral inguinal lymphadenectomy on SS transplanted animals meant a non significant reduction in lymph-node metastases In AS-transplanted animals, those in the lymph adenectomized group developed fewer takes in the homolateral inguinal nodes but also in more distal lymph nodes In addition, the recorded take-frequency in the lungs tended to be reduced ($p < 0\ 10$ by X^2 test)

Study B Again the AS tumours were larger than SS at amputation ($p < 0\ 05$ after 2 weeks, $p < 0\ 001$ after 3 weeks) and the SS tumour was killing the hosts more slowly than AS The general pattern of metastases followed that in Study A Thus, while AS had, at most, 50 per cent detectable lung tumours, all dying animals had gross extrapulmonary metastases These were mainly found in lymph nodes (contralateral inguinal, lumbar and axillary) and muscle A surprisingly high frequency of heart metastases was recorded (in 12 of 14 positive animals in Group II, Table 4) The grossly recorded number of myocardial metastases may be rather exaggerated since the tumour nodules in the heart often were seen to be confluent in histological sections There was also a rather high frequency of other metastases to muscle (9/14 mice in Group II Table 4)

Conversely, animals dying from SS metastases had very large lung tumours (cf mean lung weights in Table 3), but only few extrapulmonary tumours

COMMENTS

The present studies demonstrate the usefulness of radically resectable tumours in meta stasis experiments As pointed out in a previous review (*Hagmar* 1970), the radical removal of the primary tumour prolongs the survival time of the hosts and permits the accurate assessment of presence and distribution of metastases Bioassay procedures

(*Goldie et al* 1953, *Donelli et al* 1969) may give similar results, but are far more tedious and expensive

In Study A, all animals amputated at a certain time were killed when any animal in that group died This procedure was chosen with a view to providing a good material for histological examination since the tissues can be fixed in a viable state It will also make all animals in the group comparable, whatever parameter is studied Negative findings at autopsy, however, do not exclude the presence of disseminated, viable tumour cells Besides the advantage of verifying the results this was the main reason why Study B was conducted in which the animals were observed for spontaneous death Despite the inconvenience of observing the animals daily, this method gives the survival time as an additional parameter of "malignancy"

The AS metastases killed the hosts sooner than SS Like the quicker growth of the primary tumours in legs, this points to a greater growth capacity of the AS cell population, compared to SS The population kinetic factor-(s) that are causing this greater net growth are to be elucidated But in spite of the larger 'primaries', the AS tumour started to metastasize at roughly the same time as the SS tumour—i e, the majority of animals did not develop metastases until 2 weeks after the leg tumours were implanted Using a bio-assay procedure *Klein* (1955) and *Ringertz & co workers* (1957) found that their AS tumour started to metasize earlier than the corresponding SS tumour These apparently contradictory results need not be due to differences between the bio assay procedure in which the tumour bearing organs are implanted into new hosts, and the present procedure in which tumours grow in the original host This possibility cannot be excluded entirely however, since SS and AS will cause a specific immunity in response to their growing tumours (*Boeryd & Suurkula* 1973 *Hagmar* 1974 a) Besides, a more detailed study in the 2 3 weeks interval might give other results Yet, the basic cause is, most probably, a biological difference between two tumour host systems

Purdom et al (1958) attributed the greater metastasizing capacity in *Klein's* (1955) system to a higher electrophoretic mobility of AA cells, as compared with AS and SS cells Since the suspensions apparently have not been comparable (SS and AS mechanical suspensions with presumably low viability, AA in well dissociated viable suspensions from ascites), these electrophoretic data must be taken with a certain reservation MCG101-AA cells had also a higher electronegative surface charge density than AS and SS cells, but again the suspensions were not comparable (*Hagmar* 1974 a) Thus, any generalization concerning a greater metastsizing capacity of ascites tumours and its relationship to surface charge seems premature

In the present studies, the SS metastasis pattern differed consistently from the AS pattern The SS hosts died from large lung tumours and metastases outside the lungs were rather scarce On the other hand, AS hosts died from extrapulmonary tumours The low incidence of pulmonary AS metastases may depend in part on the difficulty in demonstrating AS lung metastases histologically, especially in autolytically altered organs Nevertheless the location of the main tumour mass was extrapulmonary

Anatomical relationships (the nearest capillary system, for instance) may affect metastasis patterns to some degree, in conformity with findings in other experimental tumour systems and in man (see e g Willis 1967) Yet, the properties of tumours which otherwise determine the metastasis patterns remain unknown As explanation of the characteristic, individual 101-SS and AA metastasis patterns, any anatomical considerations may be set aside Likewise, differences in the infiltrative behaviour of the tumours, judged on the basis of the histology of the 'primaries', may be set aside

Of course, there may be quantitative differences in the cell release from the two tumours It seems more likely, however, that differences in the distribution of tumour cells

once released from the tumours may determine the different metastasis patterns If so the SS pattern should reasonably be interpreted as a retention of the majority of the released tumour cells in the first capillary bed the lungs only a small fraction being distributed to other organs On the other hand the AS metastases were not preferentially located in the lungs The reason why remains unclear Are the myocardial metastases due to a pre pulmonary filtering out of tumour cells which adhere to the right side of the heart before reaching the lungs? If so it could fit in with *Gasic* & *Gasic s* (1962) concept ac cording to which a slimy extraneous cell coat promotes the endothelial adhesion of tumour cells At least if growing in a serous cavity, AS and AA cells do produce a slimy so far chemically underfined material But AS me tastases arose also at other muscular sites for instance in the chest abdominal wall dia phragm etc. In these cases the tumour cells must have passed the lungs before they developed into tumours This may also be true of the myocardial metastases which might reach their target via the coronary arteries In this case—perhaps in any case—the tumour might be called myotrophic Are surface recognition sites on tumour cells retaining the cells in certain organs or vessels like they seem to do for lymphocytes (*Woodruff* & *Gesner* 1968)? Other results indi cating that cell surface characteristics may play a role as determinants of metastasis patterns (*Hagmar* 1970 *Hagmar* & *Norrby* 1973) make this explanation attractive Yet in this case it is probably not the only ex planation since intravenously injected AA cells almost exclusively gave rise to pulmo nary tumours (*Hagmar* 1974 b) On the con trary and in contrast to the present findings intravenously induced SS metastases were frequently encountered in extrapulmonary organs (*Hagmar* 1974 b)

Except in muscle both tumours especially AS produced metastases in lymph nodes If the homolateral right lymph node was left intact the metastases would be located there with or without more distal foci This favours the view that the lymph node metastases re present a true lymphatic spread and not as discussed above a preferred site for blood borne metastases If the closest (inguinal) lymph node was removed Group IV Study A) the incidence of other metastases would fall, but not significantly in any particular organ Lymph node metastases were in these cases and in Study B sited more proximally along the iliac vessels but also in the axillae In Study B homolateral inguinal lymphade nectomy was performed on all mice in an attempt to prolong the survival times and to prevent secondary spread from involved nodes As mentioned the metastasis para meters (where comparable) differed very little from those in animals in Study A which were not lymphadenectomized the only dif ference being manifest in the frequency of takes in the right inguinal node itself Thus a number of the removed nodes must have contained tumour cells a conclusion also verified histologically in some cases (not shown in the tables) Perhaps these data from a tumour of defined antigenicity (*Boeryd* & *Suurküla* 1973 *Hagmar* 1974 a) will add some information in the current debate about the protective role of lymph nodes in relation to tumour spread (see e g *Bard et al* 1969 *Crile* 1969)

Yet and of the greatest importance the present results should initiate new stud es (i) to learn wether there is a general pattern behind the effects of ascites transformation upon metastasis formation and (ii) to match a specific metastatic behaviour v th proper ties of the tumour cells especially with their surface characteristics

The sk lful techn cal ass stance of M ss *Marianne Brenner* and Mrs *Lena Sandahl* is gratefully ac knowledged

The study was supported by the *Swed sh Cancer Society* (no 465 B72 03X) and *Goteborgs Lakar sallskap*

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PSEUDODIPLOID KARYOTYPE IN A BREAST CARCINOMA

A Case Report

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One case of a low differentiated breast carcinoma showed a well defined karyotype with loss of one chromosome No 16 and an additional chromosome in the B group

Several studies have in recent years described different numerical and structural abnormalities of the chromosomes from breast carcinomas (*Makino et al* 1959, *Miles* 1967, *Ishihara et al* 1961, *Hauschka* 1963, *Ishihara et al* 1963, *Makino et al* 1964, *Jackson* 1967, *Katayama* & *Masukawa* 1968, *Toews et al* 1968, *Messinetti et al* 1971) Most of them were carried out from malignant effusions The investigations of solid mammary carcinomas have been relatively few due to the fact that the preparation of chromosomes from the solid tumour presents numerous technical problems On the basis of earlier analyses, the chromosome number showed a wide range of variations and the cells had different structural abnormalities, such as fragments rings morphologically distinct marker-chromosomes multiradial configurations etc Only one case of breast carcinoma with pseudodiploidy has been published by *Messinetti et al* (1971) who claimed having found a connection between the male karyotype and the tumour cell karyotype Another well defined karyotype from a breast carcinoma is presented in this paper

MATERIAL AND METHOD

Case report The patient was a 62 year old female first operated upon in 1953 for a mammary carcinoma with local excision of the tumour and then with exaeresis axillae No lymph nodes were involved Postoperatively she was given X irradiation as a routine measure

In 1972 she developed a solid tumour in the surgically treated area, and the tumour was then excised A piece of this tumour was taken for chromosomal study The pathological diagnosis showed a low differentiated carcinoma and at a later stage the patient developed metastases in the skeleton

Chromosomal processing The piece from the tumour was minced in Earle Eagle s medium with fetal calf serum to which colchicine had been added to a concentration of 1 μg/ml After incubation at 37° C for 2 hours chromosomal preparation was made by the airdrying method The control was set up from leukocyte culture The simple Giemsa staining was carried out Attempts for chromosome banding were unsuccessful

RESULTS

The chromosome analysis of the tumour was based upon 25 suitable metaphases Fig 1 shows this karyotype with the deviation,

Received 18 x 73 Accepted 4 xii 73

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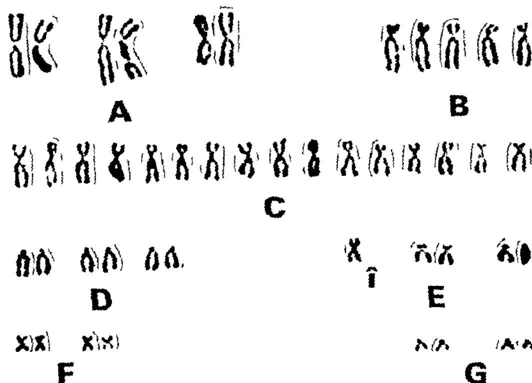


Fig 1 Pseudodiploid stem line karyotype of a low differentiated breast carcinoma 46, B+, E—

which was found in all metaphase plates the absence of a chromosome No 16 (see the arrow) and an additional chromosome in the B group Each of the cells contained 46 chromosomes The control analysis of the leukocyte culture from this patient showed a normal diploid karyotype

DISCUSSION

Malignant tumours with a uniform chromosomal picture are rare The solid tumours are generally characterized by aneuploid chromosomal constitution Deviation from the normal chromosomal set is variable, with a range stretching from hypodiploidy to hypertetraploidy This great variability is often observed within the same tumour connected with different abnormal chromosomal morphology (*Koller* 1972) *Mesmnetti et al* (1971) have published a female breast carcinoma which showed diploid chromosomal constitution, *e g* the absence of one member of the C(6–12)+XX group and the presence of an additional chromosome, which was morphologically very similar to the Y chromosome The present case stands in contrast to the above mentioned general observations The cells consistently showed the loss of chromosome No 16 and a surplus in group B The controlling chromosomal investigation from leukocyte culture showed a normal karyotype, so that the leukocyte-origin of abnormal cells proved to be improbable

An explanation is the assumption that one and only one cell from the primary tumour has given rise to this abnormal stemline On the basis of the above material, and that of *Mesmnetti et al* (1971) clearer information

about breast tumours is beginning to be obtained

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EXTRACORPOREAL IRRADIATION OF THE BLOOD

Histological Examinations of Homografts in Patients Undergoing Immunosuppressive Therapy with ECIB before Kidney Transplantation

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By means of extracorporeal irradiation of the blood (ECIB) lymphopenia is produced in man This method has been used in the clinical work as immunosuppressive therapy before and after renal transplantation In the present study, the histological changes of acute and chronic rejection in non surviving kidney grafts were compared in 29 patients who received ECIB before and after grafting (group I) and 42 non ECIB treated patients (group II) The median survival time of the graft was 45 days (range 2-790) In addition, the histological changes in biopsies from the grafts taken during early acute rejection episodes were studied The histological changes were divided in acute rejection and chronic changes and graded according to the severity of the observed changes Furthermore, the degree of mononuclear cell infiltration were evaluated No statistical difference between group I and II, neither regarding histological changes in the grafted kidneys nor in the biopsies, could be demonstrated

Based upon experimental work, depletion of small lymphocytes in the organism may weaken the transplantation reaction (*Gowan & McGregor* 1965) Among other methods extracorporeal irradiation of the blood (ECIB) has proven useful in the production of a pronounced and prolonged lymphopenia in uraemic patients, (*Weeke* 1973) and has been used as immunosuppressive therapy before and after transplantation (*Cronkite & Chanana* 1968, *Persson et al* 1971, *Weeke & Thaysen* 1973 a) Thus, patients who have received ECIB before and after renal transplantation display fewer rejection episodes than non ECIB treated patients This effect seems, however, not to influence significantly long term graft survival, at least if all kinds of failures, "immunological" as well as "non immunological", are considered (*Weeke & Thaysen* 1973 b) Histological examinations of the grafts after pretransplantation therapy with ECIB may therefore be relevant and is the purpose of the present study

Received 17.xii 73 Accepted 17.xii 73

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MATERIAL AND METHODS

Patients In the period between December 1968 and December 1973 a total of 143 consecutive, first necro-kidney transplantations have been performed Three patients were excluded, one because it was a 4 year-old child, and two because they were transferred to another hospital after transplantation

Among the remaining 140 patients were 73 patients with non surviving grafts, a further 2 of

TABLE 1 *The Clinical Data of the 71 Patients with Non-Surviving Grafts (Median Values and Range)*

Group	No of ptt with non surviving grafts	Recipients			Match Grades				Donors		
		Age (years)	Sex	Observation time	A	C	D	E	Age (years)	Ischaemic period warm (min)	Ischaemic period cold (h)
I + ECIB	29	42 (24–56)	13 ♂ 16 ♀	45 days (6 days–22 m)	2	9	17	1	38 (15–63)	15(0 90)	6¾(½–16)
II − ECIB	42	47 (15–57)	18 ♂ 24 ♀	55 days (1 day–23 m)	2	25	15		27 (6–62)	18(0–50)	6½(2–20)

TABLE 2 *The ECIB Schedule before and after Transplantation (Median Values and Range)*

No of patients	Before transplantation			Period between end of ECIB and transplantation (mo)	After transplantation			
	Transit dose (rads)	Total dose (rads)	Hours		Transit dose (rads)	Total dose (rads)	Hours	Days after transplantation
6	100 (14 105)	20500 (6680–39900)	91 (54–69)	7 0 (⅓ 12)	300 (100–300)	27250 (15540–88370)	30 (22 115)	1 30 days
23	340 (260–650)	51000 (2340 76600)	74 (51–74)	4 5 (1/30–14)	300 (44–550)	36600 (1570–72450)	57 (3 83)	1 21 days

these were excluded from the present study because histological specimens from the grafted kidney were not available

Among the remaining 71 patients with non surviving grafts, 34 died and 37 had the graft removed The median age of the patients, 32 men and 39 women, was 45 years (range 15–61) Twenty nine of the 71 patients received ECIB before and in the period immediately after transplantation (group I), 42 did not receive ECIB before transplantation (group II) Five of the patients in group II received ECIB after transplantation only in connection with rejection episodes In Table 1, the clinical data of the recipients and donors in the two groups are shown No significant difference between group I and group II could be found ($p > 0.05$)

Tissue typing of all recipients and donors were done before transplantation in the tissue typing laboratory of Rigshospitalet by L Staub Nielsen and A Svejgaard according to the methods of *Kissmeyer Nielsen & Thorsby* (1970 a) The match grades are presented in Table 1 as A, B C, D and E match according to generally accepted criteria (*Kissmeyer Nielsen & Thorsby* 1970 b)

The transplantations were performed by conventional technique, the donor kidney being placed in the contralateral iliac fossa The age of the donors as well as the ischaemic periods of the donor kidneys in group I and II are shown in Table 1 In group I, 6 donors were more than 50 years old and in group II, 7 were older than 50 years The ischaemic period was prolonged (warm ischaemic period more than 60 minutes and/or cold ischaemic period more than 10 hours) in 6 cases in group I and in 7 cases in group II

Fig 1 Acute rejection with interstitial oedema and mononuclear cell infiltration Arterioles show endothelial swelling (Haematoxylin-eosin × 182)

Fig 2 Arteriole with endothelial swelling and oedema Lumen obliterated (Haematoxylin-eosin × 350)

Fig 3 Arteriole with endothelial swelling and oedema Vessel wall shows fibrinoid necrosis (Haematoxylin eosin × 350)

Fig 4 Two branches of the renal artery showing fibroblast proliferation and lipid containing histiocytes in intima (Elastin stain × 140)

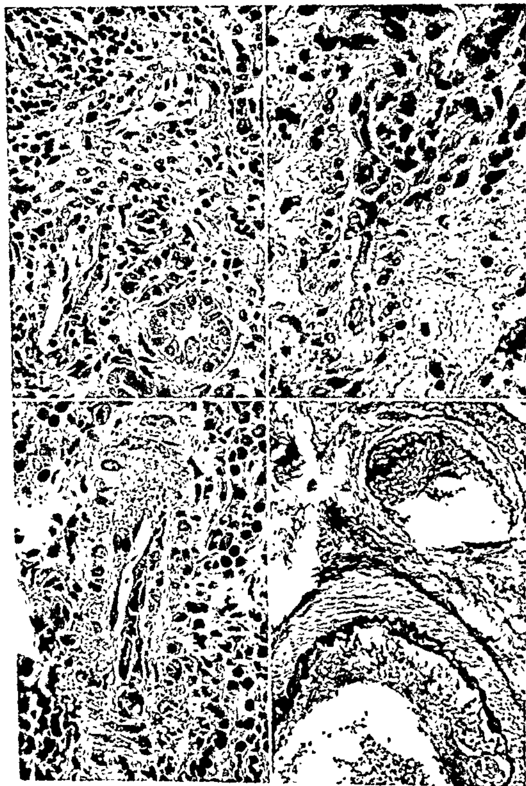




Fig 5 Branch of renal artery with pronounced fibrous thickening of intima (Elastin stain × 70)

ECIB The technique of ECIB and the irradiators have been described in detail elsewhere (*Weeke* 1972 a) In Table 2, the ECIB schedule before and after transplantation applying to the 29 ECIB treated patients in group I is shown

The immunosuppressive therapy after transplantation consisted of azathioprine and prednisone ejection crises were treated with increased dose prednisone Twelve patients, all in group II, received local graft irradiation (150 rads 3 times during the first week after transplantation)

The clinical course after transplantation is presented in Table 3 Acute rejections were diagnosed according to generally accepted criteria as described elsewhere (*Weeke & Thaysen* 1973 a) It appears that 6 patients in group I and 5 patients in group II had no clinical rejection episodes They all died of causes unrelated to the grafted kidney

Histological examinations of all the grafts were performed when they were removed or after the death of the patients Furthermore, biopsy of the grafted kidneys was performed during acute rejection episodes in 10 of the 16 patients in group I and in 14 of the 27 patients in group II

Sections of paraffin imbedded tissue stained with haematoxylin-eosin, PAS and according to van Gieson Hansen were reviewed blindly In order to eliminate previous fibrous and vascular changes, histological changes in biopsies and grafts were compared to the findings in the initial biopsy of the donor kidney, if available (37 of 71 cases) Immunofluorescence studies were not performed

The histological changes were classified as follows
Hyperacute rejection
Acute rejection, grade a–c
Chronic rejection, grade 1 3
Vascular insults

The criteria for the classification was based on changes in human allografts, described elsewhere (*Busch et al* 1971, *Dammin* 1966, *Herberton* 1969, *Kissmeyer Nielsen et al* 1966, *Olsen et al* 1966, *Porter* 1963 and 1964)

Hyperacute rejection

Microthrombosis in glomeruli, partial or total cortical necrosis without thrombosis of renal artery or vein No interstitial infiltration

Acute rejection:

Grade a Interstitial oedema and mononuclear infiltration Slight to moderate arteriolar endothelial swelling (Fig 1)

Grade b Interstitial changes as a, severe endothelial swelling and oedema leading to almost complete obliteration (Fig 2)

Grade c Changes like a and b with fibrinoid necrosis of arteriolar wall and/or thrombosis Microthrombosis might be present in the glomeruli (Fig 3).

The vascular changes in acute rejection affect primarily arterioles, but may in severe cases also involve branches of the renal artery

Chronic rejection, or fibroproliferative arterial changes

Grade 1 Intimal endothelial and fibroblast proliferation in interlobular arteries and major branches of the renal artery Slight mesangial and interstitial fibrosis without glomerular obsolescence or tubular atrophy (Fig 4)

Grade 2 Fibrosis of arterial wall Slight to moderate tubular atrophy and interstitial fibrosis Mesangial fibrosis, but no glomerular obsolescence Infarction may be present (Fig 5)

Grade 3 Pronounced fibrosis with tubular atrophy and glomerular obsolescence

Vascular insults Thrombosis in major branches of renal artery or vein

RESULTS

Histological examination of the grafts after removal or at the patient's death Table 3

TABLE 3 *The Clinical Course and the Histological Changes in the 71 Patients (Median Values and Range)*

roup	No of patients	per cent	Clinical course	Day of graft examination	none	Histological changes: acute rejection a	b	c	chronic changes 1	2	3	Other*
	7	24 5	non viable	9 (2–15)	1		1					5
ECIB	16	55	acute rejections	50 (6–660)	2	2	4	2	3	3		
	6	20 5	*no rejections*	112 (28–300)	2				4			
otal	29	100		40 (2 660)	5	2	5	2	7	3	0	5
	10	24	non viable	10 (1–33)	2	1	1	2				4
ECIB	27	64	acute rejections	66 (7–780)	1	4	4	2	9	7		
	5	12	no rejections	47 (7–790)	2				1			2
tal	42	100		57 (1–790)	5	5	5	4	10	7	0	6

Hyperacute rejection and vascular insults

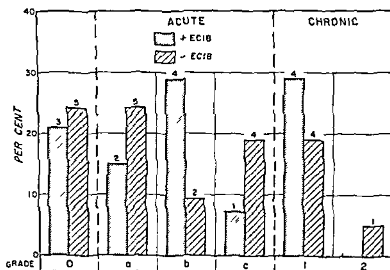


Fig 6 The histological changes in grafts which were removed within the first 2 months after transplantation in 14 ECIB treated patients and 21 non-ECIB treated patients Abscissa histological changes graded as described in text Ordinate per cent The number of patients is indicated on the top of each column

shows the histological changes in the non surviving grafts applying to the ECIB treated group I and the non-ECIB treated group II The differences were not significant ($p > 0 1$)

Eighteen patients in group I (64 per cent) and 27 patients in group II (63 per cent) lost the grafts or died within the first two months after transplantation The relative distribution of the histological changes among these patients are shown in Fig 6 No statistical differences between the two groups could be demonstrated ($p > 0 1$)

Histological examinations of biopsies taken during acute rejection episodes The histological changes in biopsy specimens taken in

TABLE 4 *The Distribution of Clinical and Histological Rejections among 29 Patients in Whom Biopsy on the Graft was Performed during Acute Rejection Episode*

Group	No of patients	per cent	Days after transplantation of clinical rejection episodes	Days after transplantation of histological examination	Histological changes none	acute rejection a	b	c
I + ECIB	11	38	7 (5 45)	23 (5–46)	1	5	4	1
II — ECIB	18	43	14 (3–180)	15 (5–180)	1	7	5	5

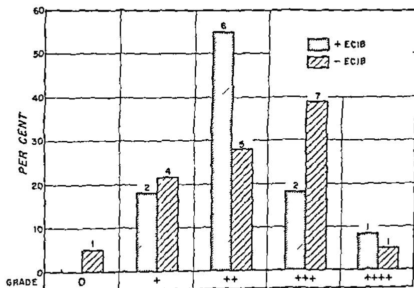


Fig 7 The degree of mononuclear cell infiltration in biopsies taken during acute rejection episodes in 11 ECIB treated patients and 18 non ECIB treated patients Abscissa Degree of cell infiltration Ordinate per cent The number of patients are indicated on the top of each column

close relation to acute rejection episodes (10 in group I and 14 in group II) as well as in 5 non survival grafts (1 in group I and 4 in group II) that did not function due to acute rejection are shown in Table 4 No significant differences could be found (p > 0 1)

In Fig 7 the degree of lymphocyte infiltration in these biopsy specimens are shown The differences were not significant (p > 0 1)

DISCUSSION

The effect of ECIB in production of lymphopenia both in animals and patients seems established (*Cronkite* & *Chanana* 1968, *Weeke* 1972 a and *Weeke* 1973) Pre transplantation therapy with ECIB may therefore be expected to weaken the transplantation reaction, including the histological changes of acute and chronic rejection

In acute homograft reaction the morphological changes in the grafts are characterized by interstitial mononuclear cell infiltration oedema and arteriolar changes in the form of endothelial swelling fibrinoid necrosis and/or mural thrombosis Complete repair may occur if sufficient immunosuppressive therapy promptly is instituted (*Porter* 1964)

In the late homograft reaction the morphological changes differ from those seen in

acute rejection (*Busch* 1971) Chronic obliterative arterial lesions, which term is used for late reaction, involve primarily larger branches of the renal artery and lead to interstitial fibrosis, tubular atrophy and glomerular obsolescence These changes are most often found in long-term survivors who display no clinical signs of rejection episodes, but may also be a consequence of severe acute rejection involving branches of the renal artery

Previous histological studies of grafts after ECIB treatment are rather sparse In cattle receiving ECIB before skin grafting, the grafts displayed only minimal mononuclear cell infiltration (*Chanana et al* 1966) In two patients who after transplantation received ECIB only as supplement to conventional antirejection therapy, examination of the grafts displayed minimal to moderate mononuclear cell infiltration (*Maginn & Bullimore* 1968) Histological studies of kidney grafts in animals or patients, after pre- and post-transplantation therapy with ECIB have not been performed systematically before

In the present study, histological examinations of the grafts, after removal or at the death of the patients, display an equal distribution of the acute and the chronic rejections as well as of the severity of these changes among the ECIB treated patients and the non ECIB treated patients (Table 3) The great variability in the periods between transplantation and examination of the grafts may however, influence the present results and make it difficult to draw any conclusions

Meanwhile, biopsies of the donor kidneys were performed during acute rejection episodes in about one third of the patients The rejections occurred in all but one patient within the first six weeks following grafting As regards the patients in groups I and II, however, the histological changes in these biopsies were not significantly different either regarding the severity of acute rejection, or the degree of mononuclear cell infiltration (Table 4 and Fig 7)

The most likely explanation is that the lymphopenia produced during ECIB is limited to ¼–⅓ of the pre ECIB level (*Weeke* 1973) Furthermore, the lymphocyte destruction seems to be unspecific, involving both T- and B-lymphocytes (*Weeke* 1972 b) It might therefore be assumed that a sufficient number of specifically committed lymphocytes remains alive and normally functioning after ECIB, and thus are able to induce the transplantation reaction This is in accordance with the *in vitro* finding of an unchanged mixed lymphocyte reaction per unit number of lymphocytes during and after ECIB (*Weeke* 1972 b)

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HISTOLOGICAL AND FOLLOW-UP STUDIES IN CASES OF MICRO-INVASIVE CARCINOMA OF THE UTERINE CERVIX

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Two hundred and ten cases of suspected micro invasion or definite micro invasive carcinoma of the uterine cervix (i e penetration not beyond 5 0 mm) were histologically subdivided according to the classification proposed by *Frick et al* (1963) This classification was found to be of no value as a means by which to predict the final outcome of the disease in patients in the present series The cells in micro invasive foci often showed signs of cytoplasmic maturation Stromal lymphocytic infiltration was increased as compared with that in cases of carcinoma *in situ* Oedema like halo formation was seen more often in cases in which micro invasion was deeper In the majority of the cases, suspected or micro-invasive carcinoma was associated with carcinoma *in situ* with buds In similarity with carcinoma *in situ*, micro-invasive carcinoma was found to occur predominantly in premenopausal patients The follow up periods ranged from 6 to 18 years in all but one case Twelve per cent of the patients were treated only with conization no sign of recurrence has yet been detected in these patients Despite the limitations of the technique, measurements of the extent of the vertical invasion in tissue sections appear to be a method by which a good prognosis may be predicted after treatment of patients with clinical Stage I carcinoma of the uterine cervix

A great deal of literature concerning the histological appearance of carcinoma *in situ* of the uterine cervix has been published By contrast, little information on the histological features of micro invasive carcinoma in that organ has appeared The majority of the reports dealing with micro invasive carcinoma give no description of the histological details found in tissue sections (*Wheeler* 1956, *Dilworth* & *Maxwell* 1962 *Levitt* & *Rubin* 1965 *Scott et al* 1966 *Roman* & *Latour* 1967 *Way et al* 1968 *Kolstad* 1969 *Boyes et al* 1970 *Boutselis et al* 1971 and *Park et al* 1973

Received 23 xii 73 Accepted 23 xii 73

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According to different authors the rates of incidence of microinvasion in cases of otherwise predominantly intra epithelial carcinoma of the cervix varies from 3 per cent (*Friedell et al* 1958) and 6 per cent (*Fidler* & *Boyes* 1959) to over 40 per cent (*Moore* 1961) *Latour* (1961) reported an even higher incidence, namely 50 per cent These significant variations in reported results probably reflect wide differences in the definition of micro invasive carcinoma

According to *Mestwerdt*, who in 1947 introduced the concept micro-invasive carcinoma *per se*, the limit for the penetration depth of the infiltrating carcinoma was defined to be not greater than 5 mm from the basal membrane *Frick et al* (1963) subdivided micro-invasive carcinoma into Ia_0 (an in-

determinate group of patients with suspected micro invasion), Ia_1 or minimal invasion (penetrating no more than 3 0 mm in depth), and Ia or superficial invasion (penetrating more than 3 0 mm but not over 5 0 mm in depth) In later years, several authors (*Foushee et al* 1969, *Mussey et al* 1969, and *Marcuse* 1970) have followed the classification introduced by *Frick et al* Lesions with definite but limited (3 0 mm or less) invasion of the stroma have been considered to be of less serious importance by *Mussey et al*

In order to gain information on the biological significance of the *Frick et al* classification, we analysed a series of 210 patients with suspected micro invasion or micro invasive carcinoma long term follow up of these patients was included The results of this study are reported below Information regarding the types of associated intra epithelial lesions as well as the changes found in the stroma in suspected or definite micro invasive areas is also given

MATERIAL AND METHODS

The records of 1740 patients with stage I carcinomas of the cervix treated at Radiumhemmet between 1954 and 1966 (Table 1) were reviewed The routine histo pathological data found in 249 of these records suggested suspected or micro invasive carcinoma. Clinical examination of the uterine cervix revealed no gross lesion in any of the 249 patients A critical review of routine histological sections from all 249 patients demonstrated the presence of suspected micro-invasion or micro-invasive carcinoma in 194 cases the remaining 55 patients were excluded from the present series because the maximum depth of the invasion foci exceeded 5 0 mm During the review of 547 cone specimens reported originally as carcinoma *in situ* 16 additional cases of suspected or micro invasive carcinoma were detected Thus 210 cases of suspected or micro-invasive carcinoma were included in the present series (cf Table 1)

Punch or wedge biopsies were performed *in all* 210 cases conization was performed in 94 of these cases and hysterectomy in 6 cases (hysterectomy was preceded by conization in one of these cases)

Radiotherapy was administered to 181 patients to all 111 patients from whom only punch or wedge biopsies were obtained to 66 patients from whom biopsies were obtained and conization performed and to 4 patients from whom biopsies were

TABLE 1 *The Number of Patients with Stage I Carcinoma of the Cervix Treated at Radiumhemmet between 1954 and 1966*

Year	Total no of cases stage I	Microinvasive carcinoma Ia_0	Ia	Ia_1	Total (percentage)
1954	122	–	16	3	19 (16%)
1955	154	1	10	2	13 (8%)
1956	163	3	20	5	28 (17%)
1957	140	4	13	2	19 (14%)
1958	91	1	18	1	20 (22%)
1959	107	3	8	1	12 (11%)
1960	100		7	1	8 (8%)
1961	120	3	11	1	15 (13%)
1962	140	1	7	3	11 (8%)
1963	129	2	10	4	16 (12%)
1964	113	3	9	1	13 (8%)
1965	131	1	8	1	10 (6%)
1966	80	4	20*	2	26* (19%*)
All years	1 740	26	157	27	210 (12%)

* 4 cases treated early in 1967 are also included
(Patients with micro invasive carcinoma who died of disseminated cervical cancer 3 treated in 1956 1 in 1958 1 in 1962 1 in 1963 and 1 in 1964)

obtained and hysterectomy performed Of the remaining patients to whom radiotherapy was not administered, 25 were treated with biopsies and conization 2 additional cases with biopsies and conization followed by local electrocoagulation, 1 by hysterectomy following biopsies and 1 by hysterectomy following biopsies and conization

Histological Classification

The lesions were classified according to *Frick et al* (1963) into suspected micro-invasion (Ia_0) and micro invasive carcinoma (Ia_1 and Ia_2) Cases of micro invasive carcinoma were subdivided into Ia_1 if invasion had penetrated up to 3 0 mm in depth and Ia_2 if invasion had penetrated 3 1 mm but not beyond 5 0 mm Measurements of the depth of invasion from the epithelium stroma border, either from the surface or from the glandular epithelium were recorded by the aid of a calibrated ocular micrometer If it proved difficult to trace the origin of the invasion, measurements were made from the epithelium stroma border of the surface epithelium Additional sections from the suspected area at the time of routine diagnosis in 29 cases of suspected micro invasion provided no additional information When the number of sections was increased—for the purpose of the present work—from a mean of 33 2 sections (range 12–46) to a mean of 117 sections (range 63 236), 3 cases of definite micro-invasive carcinoma were detected The present series therefore includes 26 cases of suspected micro invasion

Cases presenting a distinct line of demarcation between the epithelium and the stroma were excluded from the present material Cases in which the epithelium stroma border was diffuse due to chronic inflammation but in which the epithelial cells near the stroma retained a palisade arrangement were also excluded from the present series such cases are included among cases of carcinoma *in situ* (*Rubio & Soderberg* 1969 a)

Characteristics of the Intra Epithelial Lesions

The specimens were searched for the presence of associated intra epithelial lesions The highest degree of atypia was recorded As in previous publications (*Rubio & Soderberg 1969* b c and 1970) the intra epithelial lesions were classified according to the configuration of the epithelium stroma border into carcinoma *in situ* simplex (CISS) characterized by an even and straight epithelium stroma border and carcinoma *in situ* with buds (CISB) presenting epithelial buds bulging into the stroma Cases in which bud formations were doubtful were included in the former group

Host Reaction

In some cases the invasion foci and adjacent intra-epithelial lesions were underlain or surrounded either by lymphocytes or by a peri invasive oedema like halo This halo could be traversed by a few concentric stroma fibres and lymphocytes

The presence of lymphocytes was recorded when present in moderate (+ +) or in large amounts (+ + +)

RESULTS

1 *The patient*

The mean age of the patients at the time of histological detection of suspected micro-invasion (Ia_0) was 43 53 years (range of 30–61 years), in cases of micro invasive carcinoma (Ia_1) it was 43 19 years (range 24–68 years), and in cases of micro-invasive carcinoma (Ia) 45 25 years (range 31–63 years) Other studies (*Rubio & Soderberg* 1969 a) demonstrated that the mean age of 531 patients with carcinoma *in situ* was 41 34 years (41 37 years in CISS (range 25–65 years) and 41 32 years in CISB (range 24–68 years)) In 1390 cases of advanced invasive carcinoma stage I_B-IV (*Rubio et al* 1965, *Rubio* 1966) the mean age of patients was 48 59 years (range 29–68 years) The age difference between CISB and Ia_2 was significant ($p < 0\ 05$) The age difference between the group of patients with frankly invasive carcinoma I_B IV differed significantly from that in the other groups ($p < 0\ 01$) Non-statistical differences could be demonstrated in the remaining groups A certain trend showed, however, that the depth of invasion increased with age

The study of the hormonal status of the patient indicated that 20 per cent or 42 out of the 210 cases, were in menopause The number of patients in monopause was somewhat larger in the Ia_0 group than in the other sub groups, but the difference was not significant ($p > 5$ per cent) Other surveys showed that 14 5 per cent, or 77 out of the 531 patients with carcinoma *in situ* were in menopause while the rates in cases of advanced invasive squamous carcinoma of the cervix stage I_B-IV were 53 per cent (*Rubio* 1966) and 46 per cent (*Rubio et al* 1965) in 2 separate series The difference between the groups carcinoma *in situ*—micro-invasive

26*

carcinoma and advanced invasive carcinoma was significant ($p < 0.001$)

2 The Type of Specimen

Punch or wedge biopsies were done in all 210 cases Of the 112 cases having exclusively wedge biopsies 3 cases had 1 single biopsy 44 had 2 biopsies 34 cases had 3 and 31 cases had 4 or more biopsies (mean 4 8 sections)

The diagnosis of suspected micro invasion or micro invasive carcinoma was based on wedge biopsies in 113 cases (conization was performed in one of these patients) In 92 of the 94 cases in which conization was performed the presence of suspected or micro invasive carcinoma was demonstrated in cone specimens In one of the remaining 2 cases micro invasion was already present in the preceding wedge biopsies (the cone specimen showing only carcinoma *in situ*) and in the other remaining case micro invasion was found at hysterectomy following conization In 5 cases the diagnosis was established at hysterectomy (in 4 cases following multiple wedge biopsies and in one case after conization)

The number of cases of micro invasive carcinoma Ia was found to be higher in the cone hysterectomy specimens (19 out of 98 cases or 19 per cent) than in the punch wedge biopsy material (8 out of 112 cases or 7 per cent) The difference was significant ($p < 0.01$) (cf Table 2)

TABLE 2 *Type of Specimen in the Diagnosis of Suspected Micro Invasive Carcinoma in 210 Cases*

	Histology			
	Suspected micro invasion (Ia_0)	Micro-invasive carcinoma (Ia_1)	(Ia_2)	Total
At biopsy	12	93*	8	113
At cone or hysterectomy	14	64	19	97
All cases	26	157	27	210

* One patient with micro invasion at biopsy was subsequently submitted to conization

3 Characteristics of the Areas with Suspected or Micro Invasive Carcinoma

Suspected micro invasion (Ia_0) was characterized by epithelial alterations generally of carcinoma *in situ* type with absence of visible epithelium stroma border and with *spike like irregularities in the epithelium near* the stroma The palisade arrangement of the cells near the stroma was lost These structural alterations were strongly under suspicion for micro invasion although definite signs of micro invasion were not in evidence

Micro invasive carcinoma (Ia_1 and Ia_2) was characterized by the presence of definite stroma invasion not beyond 5 0 mm in depth The invasion focus could originate in the surface epithelium or from the glands involved in adjacent areas displaying intra *epithelial atypias usually of carcinoma in situ* type In other cases the origin of the invasion focus could not be demonstrated despite multiple sections performed in that area

The nuclei of the invading cells had a disorganized polarity The usual palisade arrangement observed in areas adjacent to intra epithelial lesions was absent in the invasion foci (Fig 1) The maximum diameter of the nuclei in the outermost cells in such invasion foci appeared often parallel to the surrounding stroma (Fig 2) Micro invasive carcinoma was present in 184 patients (157 with Ia_1 and 27 with Ia_2)

The areas showing suspected or micro invasive carcinoma were composed of mature malignant squamous cells (i e cells with abundant cytoplasm generally with a prominent nucleolus) in 86 per cent of the 210 cases (Fig 3 and 4) In the remaining 14 per cent of cases however the micro invasive foci were composed of small undifferentiated cells (Fig 5) The proportion of cases presenting large mature cells was similar in all 3 groups investigated

In 88 per cent of the 210 cases the areas with suspected or micro invasive carcinoma were localized to the transformation zone and its surroundings exclusively to the cervical



Fig 1 Palisade arrangement in basal cells of intra epithelial lesions (top left) being altered in micro invasive foci Note oedema like halo formation as well as cytoplasmic maturation in invading foci (× 100)

canal in 5 per cent and to the portio vaginalis in 2 per cent In the remaining 5 per cent of the cases the anatomical localization of micro-invasion was difficult to evaluate

Suspected and micro-invasive carcinomas appeared to have originated from the surface epithelium in 65 per cent of the 210 cases from the glands in 24 per cent, in the remaining 11 per cent the origin could not be established

4 *Type of Associated Intra Epithelial Lesions*

The associated intra epithelial lesion in 199 of the 210 cases was carcinoma *in situ* with buds Among the remaining 11 cases 10 presented CISS and 1 severe dysplasia

The predominant nuclear configuration in the associated carcinoma *in situ* was small and round in 62 cases (30 per cent) small and elongated in 32 cases (15 per cent) and large and round in 26 cases (12 per cent) In the remaining 89 cases (43 per cent), the associated carcinoma *in situ* lesions were composed of non predominant (mixed) cell populations (see Table 3)

All 26 cases (i e 100 per cent) presenting

TABLE 3 *The Predominant Nuclear Configuration in Carcinoma* in situ *Associated with Suspected Micro Invasion or Micro Invasive Carcinoma in 209 Cases*

Nuclear type in carcinoma in situ	*Histology* Suspected or micro-invasive carcinoma Ia_0	Ia	Ia	Total
Small rounded	5	47	10	62
Small elongated	4	24	4	32
Large	5	19	2	26
Mixed	11	67	11	89
Total	25	157	27	209*

* In an additional case the lesion was associated with severe dysplasia

Fig 2 Concentric arrangement of outermost cells in micro invasive focus (× 400)

Fig 3 Cytoplasmic maturation in micro invasive foci (× 100)

F g 4 M cro nvas ve carc noma Note cytoplasm c maturation and prom nent nucleol (× 400)

F g 5 M cro- nvas e ca c noma composed of small und fferent ated cells (× 400)



Fig 6 Tumour cells in endothelium lined space (× 1 100)

predominantly large nuclei and 82 out of 89 cases (i e 92 per cent) presenting mixed nuclei population in intra epithelial lesions had mature cells in suspected or micro invasive carcinoma areas By contrast only 45 out of 62 cases (i e 73 per cent) with small rounded nuclei in intra epithelial lesions had mature cells in suspected or micro invasive areas The difference between the two former groups and the latter group was significant ($p < 0.01$) In cases presenting small elongated nuclei the rate was 81 per cent The presence of cells with long axes parallel to the surrounding stroma in areas with suspected or definite micro invasive carcinoma was observed in 56 per cent of the cases In the remaining 44 per cent, the most peripheral cells in the suspected or micro invasive areas presented an irregular polarity The proportion of cases presenting concentric arrangement was lower in suspected micro invasion (42 per cent of 26 cases) than in micro invasive carcinoma (58 per cent of 184 cases) the difference being non significant ($p > 5$ per cent) Five cases of Ia_1 and 4 of Ia micro invasive carcinoma showed tumour cells in endothelium lined spaces (Fig 6) Only 1 of these 9 patients died of disseminated cervical cancer The remaining 8 patients are doing well 6 to 13 years after treatment

5 *The Host Reaction*

In 70 per cent of the 210 cases moderate to marked lymphocytic infiltration in suspected or micro-invasive areas was observed This percentage is significantly higher ($p < 0.001$) than the 16 per cent found in 531 cases of carcinoma *in situ*

An oedema like halo surrounding invading clusters (Fig 7) was observed in 18 per cent of 157 cases of Ia_1 micro invasive carcinoma and in 37 per cent of the 27 cases of micro invasive carcinoma Ia the difference being significant ($p < 0.05$) This phenomenon



Fig 7 Oedema like halo and lymphocytic infiltration in the stroma surrounding micro-invasive foci (× 50)

was not found in cases of suspected micro-invasion

6 *Follow up*

Of the total of 210 patients 18 died between 1 and 15 years after treatment Of these 18 patients, 7 died of disseminated cervical cancer, on an average 7 0 years (range 1–15 years) after diagnosis The dissemination of cervical cancer was proven at autopsy in 6 of these 7 cases Of the 7 cases, 6 had been classified primarily as micro-invasive carcinoma Ia_1 and the remaining 1 as Ia_2

Of the remaining 11 patients out of the 18, 9 died of other malignancies, on an average 6 7 years (range 1–14 years) after the histological diagnosis The diagnoses of these 9 patients were as follows 2 had ovarial carcinoma, 2 cancer of the vagina, 2 cancer of the colon, 1 astrocytoma grade III, 1 cancer of the stomach and 1 malignant lymphoma The other 2 patients died of intercurrent diseases (one died of pulmonary thrombembolism and the other of infectious peritonitis) Autopsy was performed in all the 11 cases

Among the surviving 192 patients, 190 were followed up to 1973 Follow up of the remaining 2 patients failed after regular check-ups varying from 4 to 9 years At the time of the last consultation they were free from gynaecological symptoms and had negative vaginal smears

Three of the surviving patients displayed histological evidence of local recurrence 3, 13 and 16 years after initial therapy (two received at the time of local recurrence additional radiotherapy and the third received electrocoagulation) Two of the three patients are doing well, presenting negative cytological smears 5 and 6 years, respectively, after the last treatment, in the third case, the retreatment was completed at the end of January 1973 Three other patients had cell atypias in cytological smears One of these 3 patients was treated with electrocoagulation



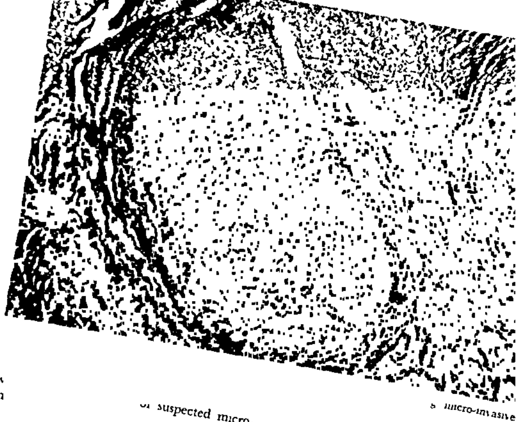
Fig 6 Tumour cells in endothelium lined space (× 1 100)

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in 1968. She is at present free from gynaecological symptoms and the cytological smears are normal. In the other 2, the smears became negative without therapy.

Of 210 cases in the present series, 25 were treated solely by conization following multiple biopsies. Sixteen of the 25 cases were detected after review of the histological preparations of 547 cases reported primarily as carcinoma *in situ*. The remaining 9 cases were originally diagnosed as suspected or micro-invasive carcinoma. Expectation after conization or refusal by the patients to accept further treatment were the causes why this type of therapy was instituted in these 9 cases.

Seven of the 25 cases were histologically classified as suspected micro-invasion and 18 as micro-invasive carcinoma (17 Ia_1 and 1 Ia). The review of the cone specimens suggested that all atypical epithelium had been removed at the time of surgery. Follow-up covered 10–18 years in 22 cases. In two other cases, follow-up covered 8 years and the remaining patient failed to appear at follow-up after 4 years. Two of the 25 patients developed local recurrence 3 and 3.5 years, respectively, after conization. They were treated in 1959 with radiotherapy. Both patients are at present doing well and have negative smears after 13 years' follow-up. Hysterectomy was performed in a third patient two years after conization because of multiple leiomyomas of the uterus. The sections taken from the cervix revealed normal histology. One year later (1969) this patient developed a squamous carcinoma in the vagina and subsequently received radiotherapy. She was seen late in 1972 at which time clinical signs of recurrence were absent.

DISCUSSION

There is much discussion about the incidence of micro-invasive carcinoma of the uterine cervix. One of the main subjects of contention has been the definition of micro-invasive carcinoma, i.e. the exact depth of stromal invasion. Some authors hold that the depth should not exceed 1 mm (*Averette* 1971), 3–4 mm (*Ullery et al.* 1965), 5.0 mm (*Mestwerdt* 1947, *Clauss & Benic* 1958, *Bangle et al.* 1963, *Frick et al.* 1963, *Morton* 1964, *Levitt & Rubin* 1965, *Margulis et al.* 1967, *Toushee et al.* 1969, *Mussey et al.* 1969, *Ng & Reagan* 1969, *Sidhu et al.* 1970, *Boutselis et al.* 1971, *Marcuse* 1971, *Artner & Holzner* 1972 and *Park et al.* 1973), or beyond 200 × power microscopic fields (*Przybora* 1965). Other authors characterize micro-invasion by the absence of confluency of the invasion foci (*Christopherson & Parker* 1964, *Boyes et al.* 1970) or by the absence of vascular involvement (*Morton* 1964, *Ullery et al.* 1965). Others have used vague histological definitions (*Govan et al.* 1966) and in some reports, the extent of the invasion has not been given (*Scott et al.* 1966, *Alousi et al.* 1967, *Way et al.* 1968, *Kyriakos et al.* 1971 and *Brudenell et al.* 1973).

One possible explanation of the reported variations in the incidence of micro-invasive carcinoma is the type of specimens in which micro-invasions have been detected, namely punch biopsy, conization or hysterectomy specimens. Unfortunately, the type of specimens has not been specified in various publications on this subject.

Another possible explanation of the reported variations in the incidence of micro-invasive carcinoma may be that the number of routine sections performed at various laboratories may vary. In the present work it was found that, by increasing the number of sections in cone specimens with carcinoma *in situ* and with carcinoma *in situ* displaying suspected areas of micro-invasion, a fair amount of definite micro-invasive carcinomas may be detected.

In the present work, 54 per cent of the diagnoses were based on punch or wedge biopsies. It may be assumed that in such cases penetration beyond 5.0 mm may have been present in the remaining cervical tissue. It should be pointed out that the diagnosis of micro-invasion in 4 of the 7 patients who died of disseminated cervical cancer was established on the basis of cone specimens.

Moreover, the high survival rate after long term follow up in the present series would seem to argue against the presence of overlooked occult foci deep in the remaining cervical tissue

Frick et al subdivided the maximum limit of stromal invasion of 5 0 mm as proposed by *Mestwerdt*, into 3 sub groups Using an objective of 2 5 × and ocular of 10 magnifications, the microscopic field was by these authors reported to be 6 mm in diameter, the half field being 3 mm This method was used to measure the depth of maximum allowed penetration (namely 5 0 mm) The method of establishing the size of penetration proposed by *Frick et al* is, however, inexact and variations of up to 1 8 mm have been found by means of different brands of microscope with various tube length Despite this snare, several authors have followed the recommendations proposed by *Frick et al* (*Margulis et al* 1967, *Foushee et al* 1969 and *Mussey et al* 1969) *Marcuse* (1971) and *Ng & Reagan* (1969) have used the same limits as *Frick et al* but measurements were performed with a calibrated microscale In the present work the histological classification proposed by *Frick et al* and based on tissue measurements as proposed by *Marcuse Ng & Reagan* was correlated with the final outcomes in a relative large number of patients after long term follow up (in 99 5 per cent of the cases follow up covered from 6 to 18 years)

In addition to the depth of stromal penetration by the tumour other histological differences have been found in cases of definite stromal invasion such as the occurrence of oedema like halos surrounding invading foci and the presence of tumour invasion in endothelium lined spaces (15 per cent of 26 cases of Ia micro invasive carcinoma and only 2 per cent of 157 cases of Ia micro invasive carcinoma significance $p < 0\,01$) These apparently important differences between the two histological types of micro invasive carcinoma were however not reflected in the final outcome of the disease in these patients the percentage of patients who died of disseminated cervical cancer was about 4 in those with Ia_1 and Ia_2 micro invasive carcinoma Moreover the proportion of local recurrences or the finding of atypical cells on smears after various modes of treatment was similar (about 3 per cent) in cases of Ia_1 and Ia_2 micro-invasive carcinoma From these considerations it would appear that the histological classification into Ia_1 and Ia_2 micro invasive carcinoma has no significant bearing on the final outcome of the disease in these patients

Suspected and micro invasive carcinoma were found to be associated in the vast majority of the cases with intra epithelial lesions of the carcinoma *in situ* of bud type Similarly to the results here obtained, marked intra epithelial atypias with buds were found to be associated with carcinoma of the cervix produced in rodents (*Rubio & Lagerlof* 1973) Moreover similar atypias with buds in rodents and carcinoma *in situ* with buds in human subjects have been seen to antedate invasive carcinoma of the cervix It is thus conceivable that intra epithelial changes with buds are severe lesions which may progress to invasive carcinoma This concept is substantiated by the histological finding that micro invasion was found to originate in the tip of the epithelial buds with carcinoma *in situ* in human subjects (Fig 8) as well as in rodents (*Rubio & Lagerlof* 1974)

It is well known that malignant tumours such as melanomas (*Lever* 1967) and mammary carcinoma (*Berg* 1959) associated with lymphocytic infiltration in the adjacent stroma have better prognoses than tumours in the same organ but without such lymphocytic infiltration In the present material lymphocytic infiltration around the area of suspected or micro invasive carcinoma occurred in 70 per cent of the cases Similar proportions of lymphocytic infiltration were found in patients who died of disseminated cervical cancer (6 out of 8 presented lymphocytic infiltration around the micro invasive foci) and in those presenting local recurrence or atypical cells in smears (4 out of 6 had lymphocytic infiltration) The effect of the lymphocytic infiltration on the prognosis of

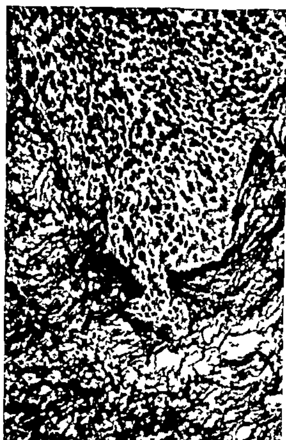


Fig 8 A group of cells breaking through the layers of reticular fibres surrounding the tip of an intra epithelial bud with carcinoma *in situ* (× 250 Lendrum s Silver stain)

micro invasive carcinoma of the cervix remains thus obscure

Of particular interest was the presence of the oedema like halo surrounding some micro invasive foci The results demonstrated that this phenomenon was significantly more common in cases in which invasion was deeper (Ia) than in those in which invasion was less deep (Ia) or micro invasion (Ia_0) was suspected In later years the presence of enzymes such as peptidases aminopeptidases and cathepsin B (*Sylven* 1973) as well as hyaluronidase (*Grossfeld* 1961) have attracted increasing interest in connection with the mechanism of invasion It has been suggested that enzymes may reduce the local resistance of the stroma and favour the infiltration by tumour cells (*Latner et al* 1973) Whether the presence of an oedema like halo surrounding invasion foci represents an increased amount of fluids containing proteolytic enzymes is at present unknown

The treatment instituted in the present series was apparently effective in arresting tumour growth in the majority of the patients concerned Eighty per cent of the patients were treated by radiotherapy 1 per cent by local electrocoagulation following conization and 1 per cent by simple hysterectomy Of particular interest is the finding that the remaining 12 per cent of the patients were treated exclusively with multiple biopsies and conization, and that they were alive and well after follow up periods covering from 4 to 18 years (as regards 24 of the 25 patients the follow up periods ranged from 8 to 18 years) These results are however, not surprising *Boyes et al* (1970) used successfully conization in the treatment of 10 per cent of 205 patients with carcinoma *in situ* with micro invasive foci and *Kolstad* (1969) obtained similar results in 4 per cent of 101 cases

The proportion of local recurrences was similar both in patients treated with multiple biopsies and conization and in patients treated either with conization and local electro coagulation or with radiotherapy or with simple hysterectomy It should be pointed out however, that in our own series of patients treated by conization the proportion of those with suspected micro invasion was higher (28 per cent) than that in cases treated with conization and local electrocoagulation radiotherapy or simple hysterectomy (10 per cent)

In conclusion, the definition of micro invasive carcinoma of the cervix is still a subject of debate In later years, however the maximum limit of 5 0 mm has been adopted by many authors One possible explanation of its acceptance is that it is based on objective criteria and somewhat less subjected to visual impression The maximum limit used however, may be inexact in cases of tangentially performed sections It is obvious that several parameters should be taken into account

The type of specimen and the number of sections performed should be reported. The method by which biopsies and cone specimens are sectioned should be standardized by the cutting of tissues at right angles to the mucosa and by increasing the number of sections in suspected micro-invasive areas. A calibrated ocular micrometer should be used. These premises will provide a means by which results obtained in different institutions may be compared.

Rampone et al. (1973) reported that the survival rate (corrected) in 537 cases of clinical stage IB was 86 per cent while the survival rate (corrected) in 167 cases of stage IA (i.e. less than 5.0 mm of stromal invasion) was higher, namely 97.6 per cent (*Kolstad* 1969). In a series of patients with clinical stage I cervical carcinoma treated at Radiumhemmet, the 5 years' relatively apparent recovery was reported to be 88 per cent (*Kottmeier* 1962). In the present investigation, the survival rate (corrected) in cases of lesions less than 5.0 mm of stromal invasion was similar to that in stage IA reported by *Rampone et al.*, namely 96 per cent. These results suggest that the measurement of the extent of the vertical invasion in tissue sections is a useful method by which the good prognosis in patients with clinical stage I carcinoma of the uterine cervix can be predicted.

We are indebted to doctors *P. Lagerlöf, G. Moberger, B. Tjernberg* and *P. Kolstad* (Oslo) for interest in and constructive criticism of this investigation.

The technical assistance of mrs. *K. Wennerström* and *N. von Krusenstierna* are gratefully acknowledged.

This work was supported by a grant of the Stockholm Cancer Society.

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AUTORADIOGRAPHIC STUDIES OF DYSPLASIA AND CARCINOMA *IN SITU* IN CERVICAL CONES

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Autoradiograms of histological slides of 98 cervical cone specimens with dysplasia and carcinoma *in situ* were analysed after incubation of tissue samples with ^{3}H thymidine *in vitro* There was a gradual increase in the proportion of labelled cells from normal to carcinoma *in situ* Labelling of superficial cell layers occurred in almost all sections from quadrants with carcinoma *in situ* in 80 per cent of those with severe dysplasia in 22 per cent with moderate dysplasia and in 8 per cent with slight dysplasia, while sections from quadrants with normal epithelium were autoradiographically negative in the superficial cell layers The labelling of superficial cells in slight and moderate dysplasia suggests that, despite cytoplasmic maturation, their nuclei retained the property of less mature and undifferentiated cells i e the capacity of DNA replication The highest proportion of labelled cells was found in areas with carcinoma *in situ* with buds

For many years it has been recognized that intra epithelial atypias of severe degree in the uterine cervix usually precede the occurrence of invasive carcinoma (*Te Linde* 1946, *Petersen* 1955) Similar epithelial atypias antedating invasive carcinoma have been observed in the cervix uteri of rodents after iterated application of a carcinogen (*Koprowska et al* 1958) In previous publications it was reported that invasive carcinomas of the cervix both in human subjects (*Rubio & Söderberg* 1969a) and in rodents (*Rubio & Lagerlöf* 1973a) are often associated with irregular buds of atypical epithelium bulging into the stroma Micro invasive carcinoma was demonstrated to originate at the tip of those atypical epithelial buds both in rodents (*Rubio & Lagerlöf* 1973c) and in human subjects (*Rubio & Söderberg*, in press)

In a recent study the cervical epithelium of rodents was investigated during carcinogenesis by means of autoradiography (*Rubio & Lagerlöf* b, in press) It was demonstrated that the percentage of ^{3}H thymidine-labelled cells (i e in the phase of DNA synthesis) increased correspondingly with the increased degree of epithelial atypia Autoradiographically however, some of the animals with moderately atypical epithelium behaved as severe atypias Another finding was that the highest proportion of nuclear labelling occurred in areas with intra-epithelial atypias presenting buds

In the present investigation, autoradiographic studies were carried out in the cervical epithelium of 98 women with various degrees of epithelial atypias ranging from slight dysplasia to carcinoma *in situ* The purpose of this work was to study the autoradiographical characteristics in dysplasias and carcinoma *in situ* with special attention to the labelling patterns of epithelial buds

Received 6 xii 73 Accepted 8 ii 74

MATERIAL AND METHODS

The presence of cellular alterations was detected in cervical smears in all 98 cases studied The histological diagnosis established in punch biopsies ranged from moderate dysplasia to carcinoma in situ Conization was performed in 98 cases The cone specimens were immediately after surgical removal immersed in bottles containing 50 ml Parker *199* sterile solution supplemented with antibiotics

A) *Processing of Cervical cones for Autoradiography*

The cervix was before operation labelled with a catgut suture on the top of its volar side at the point known as 12 o clock The material was trans ported without delay to the laboratory where it was cut into 4 quadrants, starting at the top of its volar side (i e 12 o'clock) The sections were made by a razor blade along the oral caudal axis at right angles to the cervical epithelium From each of the 4 quadrants, slices about 3 mm thick were cut and placed in 2 ml Parker solution to which 4 microCu ^{3}H thymidine (specific activity 6 7 milliCu/mMol) had been added as recommended by *Richart* (1963) Each slice of tissue was labelled according to the corresponding segment (*I-IV*) The preparations were incubated in a water bath at 37° C for 90 minutes in stoppered centrifuged tubes (100 ml) After the alloted time of incuba tion, the tissue slices were washed twice in saline and fixed in 10 per cent neutral formalin Nine to eighteen, 3 micrometer thick sections were made, following the oral caudal axis The preparations were coated with Kodak stripping film AR 10 and exposed for up to 20 weeks in light tight boxes at 4° C The films were developed in Kodak B19b solution for 5 min at 20° C, fixed in Kodak X ray Fix and finally stained in Harris haematoxylin during 2 minutes Alternative sections were made for conventional histological studies These were stained with H and E

B) *Histological Considerations*

The histological epithelial alterations were divided into squamous metaplasia, dysplasia (slight, moderate or severe) or carcinoma *in situ* The most severe degree of intra-epithelial alteration was recorded as characteristic of that quadrant The most severe degree of intra epithelial alteration in all quadrants was recorded as the final diagnosis As reported elsewhere (*Rubio & Lagerlof* 1973b) the thickness of the squamous epithelium of the cervix was arbitrarily divided into three equal parts BP (basal parabasal zone), IM (intermediate zone) and S (superfical zone bordering the lumen) Cytoplasm enlargement as well as decreased cyto plasmic basophilia in epithelial cells were consider ed as evidence of cytoplasmic maturation

The histological criteria were as follows (Fig 1–7) *Mild dysplasia* The full thickness of the epi thelium was composed of cytologically atypical cells, i e cells with abnormal irregular nuclei The nuclear density was larger in the BP compartment and decreased markedly towards the surface The nuclear shape in the BP compartment was rounded or elongated (with maximum nuclear diameter perpendicular to the epithelial border) The cytoplasm in the cells in the IM zone and in the S zone was large, suggesting cell maturation Cells with basal cell hyperplasia, but without atypical nuclei in the IM and S compartments, were not included in the group of mild dysplasia

Moderate dysplasia Undifferentiated cells were observed both in the BP and the IM compartments The S compartment contained atypical cells with irregular nuclei with condensed chromatin and large cytoplasm The cells on top had a direction parallel to the epithelial border The nuclear den sity decreased moderately from the BP compart ment to the surafce of the epitheilum

Severe dysplasia Two types of intra-epithelial alterations were considered among the groups of severe dysplasias a) The entire thickness of the epithelium was occupied by atypical pleomorphic cells, sometimes of bizarre appearance The cell density in the S compartment was slightly lower than that the other compartments

b) Cases with undifferentiated cells in which slightly pleomorphic, elongated nuclei reached the superficial half of the S compartment The nuclear density was similar in all three compartments ex cept that the nuclei occupying the superf cial half of the S compartment were irregular usually elong ated and pyknotic with the maximum diameter *parallel to the epithelial border* This type was considered a borderline between severe dysplas a and carcinoma in situ

Carcinoma in situ The hyperplasia of undiffe rentiated cells occupied the whole thickness of the epithelium the nuclear density being similar *in all* three compartments The uppermost cell layers however, could be flattened (up to 3 layers in thick epithelium) (*Rubio & Soderberg* 1969a) with irregular pyknotic nuclei arranged parallel to the epithelial border As in prev ous publications (*Rubio & Soderberg* 1969a and b 1970) carci

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Fig's 1–7 Haematoxylin stained autoradiographic preparations

Fig 1 Normal epithelium (× 200)

Fig 2 Slight dysplasia (× 400)

Fig 3 Slight dysplasia (note labelling in interme diate and superficial cells) (× 400)

Fig 4 Moderate dysplasia (× 400)

1

2

3

4

noma *in situ* was divided into carcinoma *in situ* simplex (CISS) and carcinoma *in situ* with buds (CISB)

C) *Recording of labelled cells* In order to record the presence of labelled cells and to quantitate the number of labelled cells in each compartment, the thickness of the epithelium was also divided into three equal parts with the aid of an ocular scale All sections were scanned and those showing the highest proportion of labelled cells were selected for quantitative determinations The percentage of labelled cells among 1 000 or more consecutive cells was recorded as the labelling index Determinations of the percentage of labelled cells were separately performed in areas with carcinoma *in situ* with buds and in those with a smooth epithelium stroma border If buds were present, the division of the epithelium into three equal parts would be somewhat difficult The following arbitrary division was therefore applied Labelled basal and parabasal cells or cells replacing those two cell layers were recorded as belonging to the BP compartment, all of the labelled cells from within the bud being alloted to the IM compartment Labelled cells from the base of the bud to the surface were classified as belonging to the S zone Cells with a nucleus overlaid by at least five grains were considered as labelled

RESULTS

Histological diagnosis Carcinoma *in situ* was recorded in 55 of the 98 cases, severe dysplasia in 8, moderate dysplasia in 15, slight dysplasia in 10 and squamous metaplasia in 4 cases Only normal epithelium was present in the remaining 6 cases

Qualitative determinations The results of the qualitative determinations of nuclear labelling in the cervix are shown in Table 1 It is seen here that labelling occurred in the BP zone in 392 blocks presenting normal epithelium, metaplasia dysplasia or carcinoma *in situ* The occurrence of ^{3}H thymidine-labelled cells in the IM zone were observed in 16 of the 34 quadrants (47 per cent) with slight dysplasia and in 172 of 173 quadrants showing moderate, marked dysplasia or carcinoma *in situ*, but in none of those with normal epithelium Thymidine-labelled cells occurred in the S zone in 3 of the 34 quadrants with slight dysplasia (9 per cent), in 7 of the 31 with moderate dysplasia (22 per cent), in 16 of the 20 with marked dysplasia (80 per cent), and in 120 of the 122 with carcinoma in situ (98 per cent) The difference in the proportion of labelled cells in the S compartment between normal epithelium and slight dysplasia was significant

TABLE 1 *Incorporation of ^{3}H Thymidine into the Various Compartments of the Cervical Epithelium in 392 Quadrants of Cone Specimens 167 with Normal Epithelium, 18 with Squamous Metaplasia, 34 with Slight Dysplasia, 31 with Moderate Dysplasia, 20 with Severe Dysplasia and 122 with Carcinoma* in situ

	Histological zones		
	BP	IM	S
Normal	167	–	–
Squamous metaplasia	18	–	–
Slight dysplasia	34	16	3
Moderate dysplasia	31	30	7
Severe dysplasia	20	20	16
Carcinoma in situ	122	122	120
Total	392	188	146

BP denotes the basal parabasal compartment, IM the intermediate compartment and S the superficial compartment



Fig 5 Severe dysplasia (× 400)

TABLE 2 *The Percentage of ³H Thymidine Labelled Cells in the 3 Zones of the Epithelium in Incubated Cones from Women with Normal Epithelium (14 Cases), Slight Dysplasia (7 Cases) Moderate Dysplasia (7 Cases) Severe Dysplasia (7 Cases), CISS (13 Cases) and CISB (4 Cases)*

	Histological zones			Total	
	Basal parabasal Per cent (range)	Intermediate Per cent (range)	Superficial Per cent (range)	No of labelled cells/total no of cells	Per cent (range)
Normal epithelium	10% (6–14)	0% (–)	0% (–)	1117/16 809	7% (6 10)
Slight dysplasia	16% (9–42)	9% (0–41)	1% (0 30)	1428/12 618	11% (6–23)
Moderate dysplasia	19% (7 29)	25% (12 33)	5% (0–36)	1470/ 8 496	17% (10–24)
Severe dysplasia	16% (8–29)	27% (11–48)	20% (6–49)	1948/ 9 468	21% (15 36)
CISS	22% (6 37)	28% (12–46)	28% (10–50)	5631/21 722	26% (14–43)
CISB	24% (14–47)	37% (31–46)	43% (37–63)	2172/ 6 334	35% (27–47)

($p<0\,01$) Significant differences between moderate and severe dysplasia ($p<0\,001$) and between severe dysplasia and carcinoma *in situ* ($p<0\,001$) were also found

Quantitative determinations The results of quantitative determinations are shown in Table 2 Normal epithelium had a mean labelling of 6 6 per cent slight dysplasia 11 3 per cent, moderate dysplasia 17 3 per cent severe dysplasia 20 6 per cent CISS 25 9 per cent and CISB 34 9 per cent There were however, large variations within each group Areas with CISB presented the highest labelling indices in all the epithelia investigated including areas with CISS in the same patient or in other patients presenting exclusively CISS The difference between CISB and CISS was statistically significant ($p<0\,05$)

The study of labelling index in the S compartment indicated that the proportions of labelled cells were significantly higher a) in severe dysplasia than in slight dysplasia ($p<0\,01$), b) in severe dysplasia and CISS than in moderate dysplasia ($p<0\,05$ and $p<0\,001$ respectively), and c) in CISB than in CISS ($p<0\,01$)

DISCUSSION

In the present report, the autoradiographic pattern of the normal squamous epithelium as well as that of dysplasia and carcinoma *in situ*, was investigated in slices of cone specimens This permitted a study of possible variations in similar lesions far from each other but incubated under the same conditions in the same case Under these conditions thymidine labelled nuclei were present in the deeper layers of the cervical epithelium

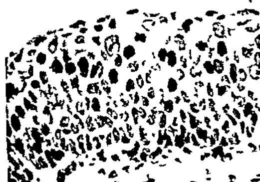


Fig 6 Carcinoma *in situ* without buds (× 200)

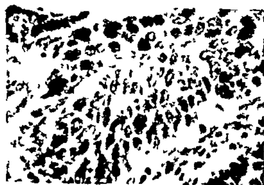


Fig 7 Carcinoma *in situ* with buds (× 200)

(BP zone) in all cases and in all quadrants presenting normal epithelium. Normal squamous cells in the IM and S zones showing signs of cytoplasmic maturation (i e with increased amount of cytoplasm and decreased cytoplasmic basophilia) demostrated absence of thymidine incorporation. *Oehlert* (1973) reported that "the detachment of the basal cells from the basal membrane appears to be linked with the loss of DNA synthetic ability". Our results are in agreement with that statement, and also with the results reported in bioptical material by *Richart* (1963), *Feitz & Syracke* (1967), *Iliya & Azar* (1967, *Kury et al* (1967) and *Schellas & Heath* (1969) in normal epithelium of the human cervix after *in vitro* incubation. Similar results were presented by *Bennington* (1973) after *in vivo* administration of ^{3}H thymidine. *Kury et al* (1967) demonstrated that the percentage of labelled nuclei in the normal stratified squamous epithelium of the human cervix is unrelated to age, pregnancy, and degree of chronic inflammation.

Several authors have reported results obtained by incorporation of ^{3}H thymidine in atypical epithelium of the cervix after incubation *in vitro* of bioptical material. *Richart* (1963) studied punch biopsies from "a group of patients" with dysplasia and carcinoma *in situ* (the number of cases was not given). He divided the epithelium into three zones: basal (only the row of cells adjacent to the basal membrane), parabasal (the lower half of the remaining thickness), and intermediate (the upper half). He concluded that the labelling index in normal epithelium and in mild dysplasia were essentially equivalent. Similar observations, namely that the superficial layers in slight or moderate dysplasias are not labelled after incubation with ^{3}H thymidine, have been reported separately by *Feit & Stanicek* (1967), *Iliya & Azar* (1967), and by *Schellas & Heath* (1969) in studies of bioptical material of 27 patients with intraepithelial atypias, including carcinoma *in situ*.

As regards the labelling pattern, some features different from those reported by the above authors in bioptical material were found in the present investigation in slices of cone specimens from patients with slight and moderate dysplasia. We found that labelled cells in the superficial zone may even occur in some cases of slight and moderate dysplasias. It should be pointed out that similar lesions within the same quadrant and in other quadrants in one and the same case might fail to show labelling in the superficial zone. This indicates that labelling of the superficial layers in some quadrants with slight or moderate dysplasia may not be an individual characteristic, but a phenomenon occurring in certain individuals in certain areas. It is thus apparent that, despite the morphologically detectable cytoplasmic maturation in slight and moderate dysplasia, some cells retain one of the properties of less mature cells and of undifferentiated neoplastic cells, namely the capacity to replicate their DNA (*Oehlert* 1973). It is thus possible that the presence of labelled nuclei in the S zone in cases of slight and moderate dysplasia reflects a disturbance in the mechanism of DNA replication in those cells (*Davidson* 1963). Histologically similar lesions thus differed in their biological behaviour in terms of thymidine incorporation. The alternative explanations of this phenomenon may be: a) that the ability of some superficial cells to incorporate thymidine represents a temporary disturbance in the DNA replication triggered by certain stimuli (i e carcinogens), b) that this behaviour may be connected with progression toward more severe epithelial changes, or c) that these lesions are in fact already severe lesions (in biological terms) despite their slight or moderate dysplastic histological pattern.

The results of the quantitative determinations suggest that the proportion of cells in DNA synthesis in the atypical epithelium increases with increasing degree of atypia from slight dysplasia to carcinoma *in situ*. Similar features have been observed in the cervical epithelium of mice treated with a carcinogen (*Rubio & Lagerlöf* 1973b and 1974a). Carcinoma *in situ* with buds have a higher percentage of labelled cells than areas with carcinoma *in situ* simplex (i e those with a

smooth epithelium stroma border) These findings indicate that the proportion of cells in the phase of DNA replication is larger in CISB than in CISS The possibility that an increased number of thymidine-labelled nuclei in the areas with CISB reflects more active biological activity in those lesions is at present being investigated in this laboratory This possibility appears to be supported by the fact that micro invasive carcinoma has been observed to originate at the tip of epithelial buds with atypias in rodents after treatment with a carcinogen (*Rubio* & *Lagerlof* 1973a and 1974b) as well as at the tip of epithelial buds with carcinoma *in situ* in human subjects (*Rubio* & *Söderberg*, in press)

Based on the results of the ^{3}H *thymidine* labelling index, *Bond* & *Johnson* (1961) proposed a formula for the calculation of the generation time in various epithelia as well as in tumours These authors chose 6 hours as "a reasonable value for the duration of DNA synthesis" both for normal and malignant cells, this time was considered valid only for cell populations of constant size in which proliferation is balanced by continuous cell desquamation Based on that formula and on the results of ^{3}H labelling index, *Richart* (1963) and *Feit* & *Stanicek* (1967) calculated the generation time in normal and atypical epithelium of the human cervix Generation time (i e the time between 2 successive cell divisions) was considered equal to the renewal time (i e the time necessary for the replacement of the entire cell population by new cells) The conclusion was drawn that the epithelium with carcinoma *in situ* may be totally replaced every 12 hours (*Richart* (1963)

On the other hand it has been established that the duration of the DNA synthesizing period, the S phase, may be quite variable both in neoplastic and in normal cells (*Baserga* 1965) Moreover only 50 per cent of the labelled tumour cells may complete cell division, and only 40 per cent of the cells in solid tumours may be actively involved in the mitotic cycle (*Post et al* 1973), the remaining cells being apparently in resting stage (*Mendelsohn* 1962)

The knowledge of the biological behaviour of the cells in intra-epithelial atypias is complicated by the fact that little is known about the rate of cell loss produced by exfoliation, the rate of cell death within the epithelium, and the direction of cell migration within the epithelium (i e vertical as against lateral migration) From these considerations it may be inferred that speculations based on the labelled index of chosen areas containing the highest proportion of labelled cells after single pulse autoradiography *in vitro* furnish an inadequate picture of the growth kinetics (in terms of generation or renewal times) applying to the whole epithelium in mucosas such as that in the uterine cervix

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The efficient technical assistance of *M Edenholm*, *Y Kock I Kranz and M Sevander* is gratefully acknowledged

We wish to express our deep appreciation to the medical and nursing staff of the Department of Obstetrics and Gynecology, Karolinska sjukhuset for their invaluable help

This investigation was supported by a grant from the *Swedish Cancer Society*

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Acta path microbiol scand Section A 82, 419-426, 1974

SELECTIVE ELIMINATION *IN VITRO* OF SENSITIVE CELL CLONES IN METHYLCHOLANTHRENE INDUCED SARCOMA BY VINBLASTINE SULPHATE

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An *in vitro* short term model was used to study whether vinblastine sulphate could selectively eliminate cells sensitive to it The effect of the drug was determined as the reduction of the incorporation of H^3 thymidine into drug treated cells compared with control cells The transplantability of the cells after incubation with and without vinblastine sulphate was examined At the transplantation of a fresh cell suspension, only a fraction of the injected cells start growing and produce a tumour When a cell suspension incubated *in vitro* for four hours is used still fewer cells survive and produce tumour growth A significant reduction in drug effect could be demonstrated in tests performed on tumours developed after *in vitro* incubation with vinblastine sulphate The most reasonable explanation of this phenomenon is that the vinblastine sulphate treatment selectively eliminates "sensitive" cells and leaves "resistant" cells alive, resulting in a change in composition of the tumour cell population

In a series of papers, *Håkansson* & *Tropé* (3, 4, 5) explored an *in vitro* technique for evaluating the effect of certain cytostatic drugs on DNA synthesis in a cell suspension prepared from methylcholanthrene-induced mouse sarcomas The technique uses the depressive effect on thymidine incorporation into DNA as a parameter of drug effect So far the technique was used to demonstrate different reactivity among tumour cell suspensions prepared from various tumour materials Thus it was possible to demonstrate marked differences in overall sensitivity among different primary tumours, a heterogeneity within such tumours, and to follow changes in these phenomena during serial transplantation of tumours to syngeneic recipients The results do not necessarily imply that the cellular response measured in the *in vitro* tests is of relevance for the *in vivo* use of the drug or the tumour response to the drug under other conditions than those used in the test

In order to more closely evaluate the possible biological significance of the effects investigated, two series of experiments were made In one, to be published presently, an effort is made to correlate the registered effect of the drugs on DNA synthesis *in vitro* with the activity of the same drug *in vivo* on a transplant of the same cell suspension as that used for the *in vitro* determination In the second experimental series, the basis of this report, the author tried to explore the *in vitro* method further with the principal object of seeing whether the cytostatic drug can selec-

Received 22 i 74 Accepted 22 i 74

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Fig 1 Autoradiogram demonstrating the uptake of 5 H^3 uridine The silver grains are mainly located over sarcoma cells. 2 800 ×

tively eliminate, from the cell suspension studied, cells sensitive to the drug in question These studies were performed with vinblastine sulphate The principle of the experiment was to graft cell suspensions after the end of the *in vitro* incubation period and to investigate whether the tumours developing from these grafts differ when incubated with or without vinblastine Ideally, incubation with the drug should eliminate all cells that can be damaged by the drug and tumours developing from the suspension should show complete resistance to the drug For various reasons, this was never achieved, but the results show a trend in this direction

MATERIAL AND METHODS

Tumour material Sarcomas were induced in syngenic female C 57 black mice by subcutaneous injection of 3 methylcholanthrene in oil (3) Tumours were brought into cell suspensions in Parker 199 medium (SBL, Stockholm) Suspensions were used for grafting and for *in vitro* incubation with or without vinblastine sulphate followed by grafting Grafting was performed as subcutaneous injections of cell suspensions with a controlled cell number (10–30 tumours in the different groups cf Table 1) Injections were made into the flanks of syngeneic animals Tumour appearance on the graft sites was evaluated by manual palpation every third day for a total of 21 days From these data graphs of tumour takes were prepared and the 50 per cent tumour take time in days was interpolated from such graphs

In vitro technique This has previously been described in detail (3) The cell suspension under study is incubated for 3 hours with vinblastine sulphate at a final concentration of 100 µg/ml or in controls with medium without vinblastine Tritiated thymidine is added to a final concentration of 2 µCi/ml and incubation is continued for a further hour The cultures are then washed with phosphate buffer, and the cells are precipitated with cold trichloroacetic acid which extracts nucleosides

TABLE 1 *Time (in Days) Needed for Appearance of 50 Per Cent of Developing Tumours after Grafting According to Cell Doses Given and Treatment before Grafting*

No of cells grafted × 10^6	Tumour number 1	2		3		4		5		6	
	no	no	C	C	V	C	V	C	V	C	V
10	3 5	3 5	8		14		6		12		8
5	3	4	9 5								
4		–	–	13 5		7 5		11		3	
2	7 5	8	11								
0 5	6 5	8	17								

no no *in vitro* incubation before grafting
C = four hour *in vitro* incubation without vinblastine before grafting
V four hour *in vitro* incubation with vinblastine before grafting

and nucleotides The amount of thymidine incorporated and the DNA content of each sample is measured as previously described (3) and an expression is formulated to estimate the amount of incorporated thymidine per DNA in the sample This expression has a logarithmic formula to make statistical calculations easier the actual formula is

$$a \quad 100 \quad \log_{10}\frac{(\text{c p m.}) \times 10^4}{(\text{AES}) \times (\text{DNA})}$$

where (c p m) is the number of counts registered (AES) is the c p m registered with automatic external standardization, and (DNA) is the amount of DNA in the sample expressed in arbitrary units but corrected for a standard curve Effects of the incubation with vinblastine indicate the difference between the mean *a* value in the three control tubes and the mean *a* value in the three tubes where cells were treated with vinblastine The difference between the log values is thus an expression of a ratio of thymidine incorporation with vinblastine to that without vinblastine

Cell viability tests Viability of the cells at the end of the incubation period was studied by grafting as described above An autoradiographic study of uridine incorporation into the cells was also performed During the last hour of incubation without vinblastine tritiated uridine 5 H^3 UR (New England Nuclear Frankfurt/M spec activity 28 4 Ci/mM) was added to a final concentration of 2 μCi/ml At the end of the experiment the cells were spun down and fixed in 60 per cent acetic acid made 1 N with respect to hydrochloric acid and then stained with ? per cent acetic orcein Stripping film autoradiography was performed according to *S I mid* 7) with Kodak AR 10 plates. Exposure time one to two weeks development with Kodak D1 ›

RESULTS

Changes in Cell Viability during Incubation without Vinblastine

When incorporation of 5 H^3 UR into RNA was studied during the last hour of incubation by autoradiography, it was found that most cells had incorporated the isotope (Fig 1) The *in vitro* incubation without any cytostatic drug thus does not markedly interfere with cell viability, as studied in this way But it is possible that injury has occurred which results in a decreased viability apparent first at a more efficient test of cell viability The transplantability of the cells was therefore tested Table 1 presents the results. Fig 2 plots the time in days taken to reach 50 per cent of tumour development against the cell number grafted Half of the material was grafted directly without *in vitro* incubation, the other half was grafted with the same cell suspension incubated for four hours without cytostatic drugs Within the interval studied, there is an approximately linear regress on in both materials For cells grafted without previous *in vitro* incubation a reduction to 10 per cent of the grafted cell number approximately results in a doubling of the time elapsing before 50 per cent of the tumours have appeared A slightly higher figure is seen for cells grafted after *in vitro* incubation, but more important, the graph for this material lies elevated over that of the former material

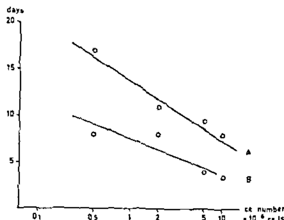


Fig 2 Plot of the time in days taken to reach 50 per cent of tumour development against the cell number grafted Half of the material was grafted directly without *in vitro* incubation, the other half was grafted with the same cell suspension incubated for four hours without cytostatic drug

The approximate 50 per cent tumour development time seen after grafting of 5×10^6 cells after *in vitro* incubation roughly corresponds to that seen after direct grafting of only 500,000 cells This would mean that only appr 10 per cent of the cells incubated for four hours without cytostatic drug show enough viability to compare with freshly suspended cells concerning grafting efficiency

Changes in Cell Viability during Incubation with Vinblastine Sulphate

Four tumours (3–6) were tested by grafting after incubation with or without vinblastine sulphate at the usual concentration 100 μg/ml In these experiments, 10×10^6 cells were grafted to each recipient after incubation with vinblastine, and 4×10^6 cells were used after incubation without vinblastine As Table 1 shows in three of these experiments, tumours developed at a very similar rate in the two series In these three series, incubation with vinblastine apparently resulted in a further loss of transplantability so that only appr 4 per cent remain transplantable after the incubation In the fourth series, the time to reach 50 per cent tumour takes was 3 days for controls and appr 8 days for vinblastine treated cells Thus a still higher percentage of cells were killed with vinblastine in this series

In vitro Reactivity to Vinblastine by Tumours Developed after Grafting

In vitro tests were performed in the standard way on cell suspensions from the primary tumours used—i e the same suspensions as those used for grafting with or without *in vitro* incubation—and on the tumours that developed after grafting Fig 3 shows the effect values registered They are expressed as differences between *a* values in incubations without vinblastine (controls) and in those with vinblastine A positive *a* value thus means a depression of DNA synthesis at the test For a comparison with this material, values are shown for unrelated tumours grafted with the same technique as that used in the present investigation Table 2 presents an analysis of variance of these data The tumour of origin does not influence the effect value seen in the grafted tumours either after incubation without vinblastine or with vinblastine ($F = 1.7$ at 6 and 43 d f, N S) On the other hand, there is probably a difference in effect values in the tumours obtained after control incubation and in those obtained after vinblastine incubation If this is studied within

0 01) but if the origin of the tumours is disregarded, a statistically significant difference of effects is present ($F = 9.8$ at 1 and 43 d f, $0.01 > p > 0.001$)

Incubation with vinblastine thus results in a reduction of vinblastine effect of the cells obtained from the tumours that develop from grafts of the incubated cells The mean effect on the tumours developing after incubation without vinblastine is 78 4 which appr corresponds to a reduction of DNA synthesis to 16 per cent when studied in the presence of vinblastine When, instead, the mean effect on the tumours developing after incubation with vinblastine is studied, it is

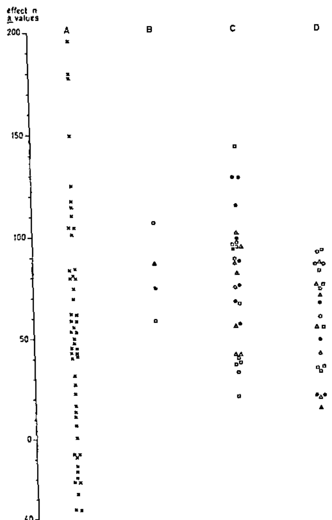


Fig 3 Diagram showing effect values for vinblastine, i e differences in *a* values for control tubes and tubes containing vinblastine A positive *a* value thus means a depression of DNA synthesis at the test
A = tumours obtained in first generation after transplantation of 52 unrelated primary tumours
B = primary tumours used for transplantation in this experiment (groups C and D)
C = tumours developed from primary tumour cells incubated without vinblastine
D = tumours developed from primary tumour cells incubated with vinblastine
Each symbol (○, ●, △, □) marks one of the four tumours in Group B and tumours developed after grafting of cells from that tumour

58 1, i e DNA synthesis is depressed to appr 26 per cent in the presence of vinblastine

DISCUSSION

The *in vitro* method used is a short term incubation The possible influence of a cytostatic drug is evaluated during a four hour period only Despite this short incubation period there are dra ti effects on the transplantability of the cells even when incubation takes place without vinblastine An approximate reduction to 10 p i ent of the transplantability has been e d d Th s does not mean that 90 per ce ot the lls studied are dead and do not contribute to the effects registered in the *in vitro* test On the contrary, autoradiographic studies of uridine incorporation into RNA during the last hour of the incubation show that most of the cells are living and metabolize We know, however, that also at the transplantation of a fresh cell suspension only a fraction of the injected cells start growing and produce a tumour When instead, a cell suspension is used that has been incubated *in vitro* for four hours, even fewer cells survive and produce a tumour

None the less, a specific change in cell composition could be demonstrated when tu-

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Acta path microbiol scand Section A 82, 427–430, 1974

CROHN'S DISEASE IN HETEROTOPIC GASTRIC MUCOSA IN A MECKEL'S DIVERTICULUM

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In a case of Crohn's disease, typical ulcerative and granulomatous changes were found also in ectopic gastric mucosa in a Meckel's diverticulum. Thus, the infrequency of gastric engagement as compared with ileo colic engagement in Crohn's disease seems to be related to differences other than those of mucosal type Possible ways of the diverticulum as such to influence the development of the disease are discussed and the incidence of a co-existing Meckel's diverticulum in Crohn's disease is commented

Crohn's disease may occur in any part of the digestive tract from the mouth to the anal region However, some parts are affected much more often than others The disease shows a predilection for the terminal ileum and colon while the stomach, for example, is rarely affected This variation in frequency of involvement between different parts of the digestive tract is probably related in some way to the still unknown pathogenesis of the disease

One favoured theory ascribes the disease to an infectious agent The variation of the microbial as well as other accused factors in the contents of different regions of the digestive tract could be responsible for the distribution pattern of the pathological changes If so the structure and defence forces of the bowel wall also have to be considered in the pathogenesis The occurrence of Crohn's disease involving a Meckel's diverticulum lined with ectopic gastric as well as ileal mucosa in the case reported below provides an opportunity to compare the pathological changes in two different mucous membranes exposed to the same environment.

Received 27 xii 73 Accepted 27 xii 73

CASE REPORT

A previously healthy 28 year-old man was admitted to hospital because of an increased erythrocyte sedimentation rate He reported that he had passed about 3 watery stools daily and had intermittent abdominal pain every day the last 18 months Physical examination showed nothing remarkable except tenderness to palpation in the right lower quadrant of the abdomen Roentgen examination demonstrated inflammatory changes in the distal part of the small bowel and the proximal part of the large bowel compatible with Crohn's disease No improvement was achieved by treatment with salicylazosulphopyridine and an operation was performed A thickened, oedematous segment was resected, including the terminal 15 cm of the small intestine and extending to the middle of the transverse colon The proximal end of the segment included a Meckel's diverticulum Many of the mesenteric lymph nodes were affected and the distal part of the appendix was hard

At morphological examination, the ileum was found to be normal at the proximal end of the specimen, but showed distally increasing changes with fissure like ulcerations into the submucosa, oedema and fibrosis in the submucosa, transmural inflam-

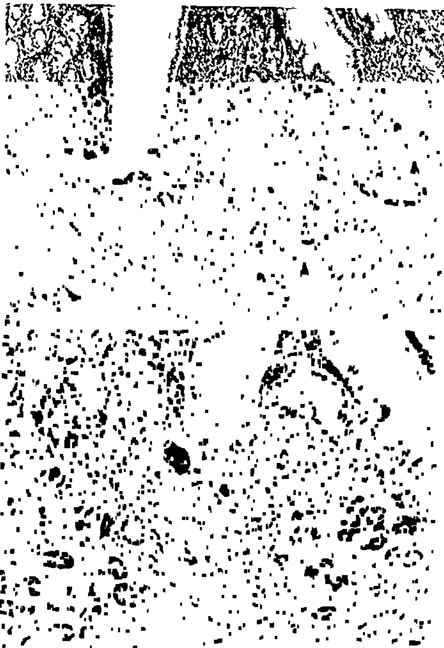


Fig 1 Gastric mucosa of fundus and antrum type Ulceration Four granulomas in mucosa and submucosa (arrows) Htx eos × 70

Fig 2 Mucosa of fundus type Early ulceration with granuloma Htx eos × 120

mation with lymphocytes plasma cells histiocytes and epithelioid cell granulomas in the submucosa as well as the subserosa (Figs 1–4) Such changes were also found in the appendix and colon In the end of the appendix, a carcinoid tumour was in evidence In the Meckel s diverticulum the mucosa was partly of ileal type partly of gastric, most ly fundic, type the submucosa, muscularis and subserosa contained pancreatic tissue with islets of Langerhan s and dilated excretory ducts The gastric as well as the ileal mucosa were ulcerated by narrow fissures coated by fibrin and polymorphonuclear leucocytes and surrounded by a plasma cellular and lymphocytic infiltration together with

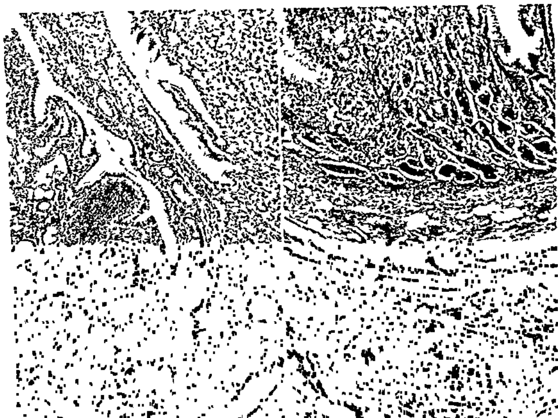


Fig 3 Granuloma in antrum type mucosa Fundus type to the right Htx eos × 70

Fig 4 Granulomas in fundus type mucosa and in submucosa Htx eos × 70

a moderate accumulation of reticulohistiocytes In the fundic, antral and ileal mucosa, in the sub mucosa and the subserosa were several epithelioid cell granulomas with giant cells and occasional central necroses Several epithelioid cell granulomas in the gastric mucosa were not associated with ulcerations The changes were far more pronounced in the diverticulum than in the adjacent ileum The mesentery lymph nodes contained granulomas

DISCUSSION

Cases of involvement of a Meckel's diverticulum in Crohn's disease of the terminal ileum are on record (1–4) and there is a single report of the disease being confined to the diverticulum only (5) To our knowledge however, this is the first report of Crohn's disease involving not only ileal, but also ectopic gastric mucosa in a Meckel's diverticulum

According to one theory, an increased permeability of the gut mucosa, passage of microbial antigens from the lumen into the bowel wall and a hypersensitivity reaction to these antigens exerted by the gut associated lymphoid tissue could be the pathogenesis of Crohn's disease(6) The difference in frequency of involvement between different parts of the digestive tract may correspondingly be related to the structures of the mucosa, to the luminal content and/or to the lymphoid tissue and local production of antibodies at different levels of the intestine

In our case of Crohn's disease involving a Meckel's diverticulum lined with ectopic gastric as well as ileal mucosa, two types of mucous membrane had been exposed to the same local environment, to the same luminal contents and to the same lymphatic drainage

Mucosal immunological defence could possibly vary with mucosal type Both types of mucosa were affected and both showed largely the same type, degree and extent of pathological changes, such as mucosal ulcerations, mononuclear cellular infiltration and granulomas It therefore seems that the type of mucous membrane, at least in this case, is of *minor importance, if any, in the development* and distribution of Crohn's disease

One might wonder whether the diverticulum as such may influence the development of the disease As for the possible role played by microbes as causal agent (7, 8), the blind pouch formed by the diverticulum may alter the microflora of the gut both quantitatively and qualitatively Another possibility is that incidents in the diverticulum may damage the mucosa and thereby increase its permeability to pathogenic agents Mechanical and ischaemic damage may result from torsion, intussusception, or foreign bodies in the diverticulum, and a chemical damage is possible when ectopic gastric tissue is present In our case, no ulcers of peptic type were found It is possible that the ectopic pancreatic juice had neutralized the peptic effect of the fundic secretion

A single report has previously commented upon the frequency of co existing Crohn's disease and Meckel s diverticulum (9) In a study of 27 cases of Crohn s disease, 5 Meckel s diverticula were found at laparotomy and it was concluded that Meckel s diverticulum was significantly more common in the series of patients with Crohn s disease than in the population as a whole We have found a similar, though less strong, tendency in a preliminary study In a series of 216 patients operated on for the first time because of Crohn's disease at Malmo General Hospital, Meckel s diverticula were noted in 13 (6 per cent), while the occurrence of Meckel s diverticulum in the general population has been given as approximately 1–3 per cent (10) However, further investigations are required to confirm this possibly increased incidence and to evaluate its relation to the pathogenesis of the disease

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Acta path microbiol scand Section A 82, 431–434, 1974

LOSS OF EPITHELIAL BLOOD GROUP ANTIGEN-A DURING WOUND HEALING IN ORAL MUCOUS MEMBRANE

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The expression of blood group antigen A was studied on oral epithelial cells during wound healing Twelve cases were studied, and the antigen reactivity was semiquantitated by titration in a double layer immunofluorescence staining reaction In eight cases there was a total loss of antigen A in the migrating epithelial cells and in the sliding cells along the wound margins In four cases there were a distinct decrease in positive reacting epithelial cells along the wound margins The positive reacting cells all demonstrated less antigen than the cells in the non wounded adjacent mucosa demonstrated by a lower end point titre The study has shown that changes in blood group antigen expression on epithelial cells during repair processes correspond to what is seen in malignant cells in oral carcinomas

It is well established that different types of human tumours have tumour associated antigens (*Gold & Freedman* 1965, *Burtin et al* 1972, *Hellstrom et al* 1971) Furthermore, certain studies indicate that the acquisition of a tumour antigen is accompanied by the loss of normal cytoplasmic membrane antigens (*Burtin et al* 1972) The expression of blood group antigens A and B is decreased or even lost in tumours developing from tissue in which such substances are normally present (*Dabelsteen & Pindborg* 1973 *Davidsohn* 1972) Although these changes have been claimed to parallel the degree of malignancy (*Davidsohn et al* 1969), they may reflect a certain surface property of normal cells When rabbit epithelial cells are grown in culture, the number of cells bearing blood group antigen A is increased with cell growth (*Franks & Dawson* 1966) Furthermore, the study of synchronized mastocytoma cells by *Thomas* (1971) has shown alterations of expression of blood group B and H antigens throughout the cell cycle

During wound healing cell movements are as active as in invasive cancers (*Loewenstein & Pen* 1967), but still part of a controlled process Wound healing is thus a suitable model to answer the question whether the loss of blood group antigen in squamous cell carcinomas is a manifestation of uncontrolled cell movement, or simply is a manifestation of cell movement in general Therefore the present study was performed in order to investigate the blood group antigens in migrating cells during wound healing

Received 20 xii 73 Accepted 20.xii 73

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MATERIALS AND METHODS

The material included twelve young healthy individuals all belonging to blood group A

Standardized wounds were made using a 3 mm





Fig 1 a Haematoxylin eosin stained wound margin 48 hours postoperative A few migrating cells can be seen (arrow) The epithelial cells adjacent to the margin are sliding towards the wound cavity (W) (× 78)
b Immunofluorescence staining of neighbouring section demonstrating an almost total loss of blood group antigen A in the migrating and sliding cells along the wound margin (A) Note the typical distribution of the antigen in the spinous cell layers of the rest of the epithelium (B) (× 78)

punch on the mucosa just inside the angle of the mouth Forty eight hours after wounding another biopsy 4 mm in diameter was taken including the two-day-old wound area and the surrounding wound margins The tissue was fixed in 10 per cent neutral formalin, embedded in paraffin, and cut as 5 μm sections

Blood group antigens in the epithelium were detected by two different immunological methods, the immunofluorescence (IF) staining and the immunoperoxidase (IP) staining Both methods were used as doublelayer techniques—blood group antiserum, conjugate, control reactions, and fluorescence microscopy were employed as described previously (*Dabelsteen* 1972) The amount of antigen in the tissue was estimated by a two fold titration of the IF staining, dilutions from 1 1 up to 1 512 were used The IP stainings were performed only with blood group antiserum in 1 2 dilutions The histology of the epithelium at the wound margins was investigated on haematoxylin-eosin stained sections

RESULTS

In all wounds the cavity was filled with fibrin and leukocytes Epithelial cell migration from the wound margins could be found in several sections In the epithelial mass adjacent to



Fig 2 a Epithelium along the wound margins haematoxylin-eosin stained (a) and immunoperoxidase stained (b)
b There is a distinct loss of reaction product in the migrating and sliding cells (A) as compared to the adjacent cells in situ (B) (× 78)

TABLE 1 *Endpoint Titres Obtained by Staining Epithelium for Blood Group Antigen A during Wound Healing*

Case number	Normal epithelium	Sliding and migrating epithelia	
		Majority of cells	Single cells
50 578	1 8	0	1 2
50 979	1 64	0	0
50 977	1 128	0	0
50 942	1 1	0	0
50 868	1 16	0	0
50 904	1 64	0	1 16
50 586	1 32	0	0
50 568	1 256	0	0
50 805	1 256	0	0
50 551	1 128	0	1 1
50 400	1 32	0	1 1
50 152	1 128	0	0

the wound margins, cells were apparently sliding towards the wound cavity (Fig 1) This was observed at a distance of approximately two rete ridges (350 μm) from the wound margin

The staining pattern was similar in the IP and the IF stainings In normal epithelium the blood group antigen was found at the cell surfaces in the spinous cell layer, the basal cells and the superficial cell layers reacting negatively (Fig 1)

In eight cases there was a total loss of blood group antigen A in the migrating as well as in the sliding epithelial cells In four cases there was a distinct decrease in positively reacting epithelial cells in both places (Fig 1–2), and the positively reacting cells all had less antigen than the cells in the adjacent normal mucosa as demonstrated by a lower end point titre (Table 1)

DISCUSSION

The re-epithelialization during wound healing is a combination of cell migration as well as sliding of the epithelial cells along the wound margins (*Fejerskov* 1973) The decrease of epithelial thickness on oral wounds along the wound margins is thus interpreted as a result of a sliding of the epithelial cell mass towards the wound cavity

The present study demonstrates that the expression of blood group antigens on oral epithelial cells taking part in wound healing is markedly decreased This decrease is very similar to what is seen in oral carcinomas and is important as the loss of blood group antigen in epithelium, in which they are normally present, previously has been interpreted as a sign of malignancy (*Davidsohn* 1969)

It is surprising that the epithelial cell mass sliding towards the wound cavity reacts negatively for blood group antigen A as probably they were all positive just before wounding

Whether this change is caused by a masking of antigen by overproduction of cell coat (*Currie & Bagshawe* 1968) or by an all over chemical change of the carbohydrate in the coat of the moving cells is not known The latter theory has recently been supported by studies on proliferating liver cells after partial hepatectomy (*Ohnishi et al* 1973)

Alteration of the cell surface membrane of neoplastic cell has been suggested as a mechanism for defective control of growth (*Abercrombie & Ambrose* 1962) That the loss of blood group antigen A in carcinomas cannot be responsible for such a defective growth control but rather is limited to cell proliferation is demonstrated by the fact that they are also lost on normal cells under conditions where these take part in considerable movements

The present work was supported by *Daell Fonden*, Copenhagen, Denmark and Grant DE 1358 from *The National Institute of Dental Research*, National Institutes of Health, Bethesda, Maryland, U S A

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EFFECTS OF SUPPLY AND WITHDRAWAL OF FLUORIDE

Experimental Studies on Growing and Adult Rabbits

3 Concentration of Acid Glycosaminoglycans and Hydroxyproline in Cortical Bone

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In one series of experiments the effect of fluoride ingestion on the concentration of hydroxyproline and acid glycosaminoglycans in the cortical bone of growing rabbits was studied During this period, excessive bone formation and resorption occurred The glycosaminoglycans were quantitatively determined using the cetylpyridiniumchloride precipitation technique in microscale and qualitatively studied by micro zone electrophoresis Concentrations of hexosamines were found to be higher in the femur than in the tibia while the contrary was true for the hydroxyproline contents Small age related changes were observed in the solubility profiles of the chondroitin sulphate No alterations were found during 14 weeks of fluoride ingestion In another series, rabbits were given fluoride during 14 weeks until completed growth after which the fluoride supplement was withdrawn and the animals followed for another 24 weeks During this period normalization of the bone morphology was established Neither quantitative nor qualitative changes occurred with respect to bone glycosaminoglycans and hydroxyproline Chemical analyses and electrophoresis indicated the presence of minor amounts of keratan sulphate in the cortical bone of rabbits

The effect of fluoride on the inorganic part of bone tissue has been extensively studied (cf *Ericsson* 1970) However, its effect on the organic constituents has been poorly investigated

Zipkin (1970) reported no changes in the ^{35}S-uptake in femur, mandibula and pelvis in fluoride treated rats despite a ten fold increase in the fluoride incorporation in the bone minerals This was interpreted as an indication 'that there was no synthesis of new polysaccharides' *Peck et al* (1965) found a reduction of total bone collagen in the calvaria of rats given 50 p p m of fluoride for one month in the drinking water

As rats are known to be relatively insensitive to the effects of fluoride (cf *Faccini* 1969), rabbits were used as experimental animals in the present investigation Growing rabbits were given fluoride in two different weight-related dosages, from 7 to 21 weeks of age when skeletal growth has ceased At that time the fluoride supplement was withdrawn The animals were thereafter followed for another 24 weeks During fluoride ingestion there was an excessive periosteal bone formation in the diaphyses of the tibae, while the morphological alterations in the femur were less pronounced (*Rosenquist* 1973 b) Although acid glycosaminoglycans constitute

Received 8 vi 73 Accepted 21 xii 73

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only a minor part of the bone matrix, important functions in bone mineralization have been attributed to them (cf *Bowness* 1968) As fluorotic bone has been described as sclerotic as well as resembling osteomalacia (cf *Faccine* 1966), it is conceivable that changes in the composition of the bone matrix could occur and possibly explain the morphological alterations

The aims of this investigation were to study a) differences in the concentration and composition of acid glycosaminoglycans and hydroxyproline in the femur and tibia of normal rabbits, b) alterations in these organic compounds during fluoride ingestion and after withdrawal of fluoride, c) correlations between chemical findings, morphological alterations (*Rosenquist* 1973 b) and actual fluoride concentration (*Rosenquist* 1973 a)

Furthermore a new attempt was made to isolate keratan sulphate from rabbit cortical bone

MATERIAL AND METHODS

Growing rabbits of both sexes and a controlled age of 48–52 days at the beginning of the experiment were fed two different basic diets called AE and L These were nutritionally equivalent (*Eriksson* 1971) and contained fluoride in unknown salt form Diet AE contained 12 p p m of fluoride and diet L 2 p p m of fluoride (for details, see *Rosenquist* 1973 a)

Diet AE was given to group I B while all other groups were fed diet L All animals were given distilled water throughout the experiment and a fluoride supplement as follows

Series I—the Effect of Fluoride Ingestion in Growing Rabbits

Group I 0 No fluoride supplement
I A 0 5 mg of F per kg body weight per day
I B 10 mg of F per kg body weight per day

Observations were made at 2 4 7 14 weeks

Series II—the Effect of Withdrawal of Fluoride after Completed Growth

Group II 0 No fluoride supplement
II A 0 5 mg of F per kg body weight per day during 14 weeks
II B 10 mg of F per kg body weight per day during 14 weeks

After 14 weeks the F supplement was withdrawn and observations were made at 4 12 and 24 weeks

The distribution of animals is given in Table 1

TABLE 1 *The Number of Animals in the Different Groups at Different Observation Times*

a) *During fluoride ingestion*

Obs times weeks	2 (9)	4 (11)	7 (14)	14 (21)
Groups				
I 0	6	10	9	9
I A	7	9	10	9
I B	8	9	10	8

b) *During the period after withdrawal of the fluoride supplement*

Observation times in weeks calculated from the day of withdrawal

Obs times weeks	4 (25)	12 (33)	24 (45)
Groups			
II 0	6	5	10
II A	6	6	12
II B	8	7	7

Fluoride Administration

Fluoride was given as NaF in a small volume of water in plastic cups When the cups were emptied the animals were supplied with distilled water

Preparation of Bone Powder

The animals were killed by an overdose of sodium pentobarbital Parts of the diaphyses from the left femur and tibia (Fig 1) were used for chemical analyses The bone marrow was removed by a sharp blast of air and the bone was rinsed in saline The bone samples thus consisted of the cortical diaphyseal bone from identical areas The samples were defatted and dehydrated in acetone changed daily for 3 days and air dried to constant weight The material was finally pulverized in a Wiley Laboratory Mill provided with a 60 mesh sieve The bone powder was stored in glass tubes

Analytical Methods

Hydroxyproline Hydroxyproline was determined on hydrolysates of roughly 3 mg of bone powder by the method of *Neumann* & *Logan* (1950) The samples were hydrolysed in 6 M HCl in sealed tubes for 16 hours at 105° C Determinations were performed in duplicate

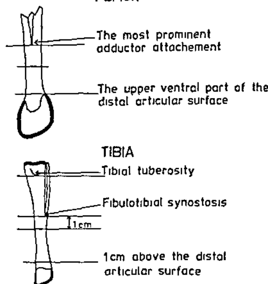


Fig 1 The bones were divided at indicated level at right angles to their long axis The middiaphysis of the left femur was divided into one proximal half used for chemical purpose and one distal half used for histochemical studies The left tibia was divided into one proximal and one distal piece both used for chemical purposes Samples from identical parts of the right femur and tibia were used mainly for morphological studies

Hexosamines Total hexosamines in bone powder were determined by the Elson and Morgan reaction as described by *Antonopoulos et al* (1964) 5 mg samples of bone powder were hydrolysed in 6 M HCl for 8 hours *Boas* (1953) method for determination of hexosamines to eliminate non specific chromogens was used in preliminary experiments but then omitted as no chromogens were found which influenced the results (cf *Larsson & Vejlens* 1969) Determinations were performed in duplicate

Quantitative determination of the CPC precipitable glycosaminoglycans CPC precipitable glycosaminoglycans were determined according to *Hjertquist & Vejlens* (1968) About 45 mg of bone powder was digested at 65° C in 9 ml digestion mixture containing EDTA cysteine HCl and recrystallized papain suspension (Worthington 40 2 mg protein ml at concentrations of 0 1 M 0 005 M and 1 per mille respectively After 4–6 hours a clear solution was obtained and the volume was brought to 10 ml with distilled water The solution was thoroughly mixed Three aliquots of 3 ml each were pipetted into glass tubes (Quickfit tubes) 4 ml of 0 per cent (w/v) aqueous cetylpyridinium chloride (CPC) solution was added and the precipitate were subsequently analysed for their hexosamine content The means of triple determinations were used

Microfractionation of glycosaminoglycans In principle, the procedure of *Larsson & Vejlens* (1969) was used About 750 mg of bone powder was digested in 50 ml of a digestion mixture containing EDTA, cysteine HCl and papain suspension at concentrations of 0 2 M, 0 005 M and 1 per mille while shaken for 4 hours at 65° C After dilution with 8 volumes of water, 50 ml of 1 per cent aqueous CPC solution was added The resulting precipitate was collected by centrifugation, washed twice in distilled water and dried The supernatant (CPC supernatant) was treated as described below

The precipitate was dissolved in 400 µl of a 60 per cent (w/v) aqueous solution of n propanol A finely dispersed residue remained undissolved and was centrifuged down and discarded From the clear supernatant 50 µl aliquots were transferred to 6 CPC cellulose microcolumns and fractionated according to *Antonopoulos et al* (1964) using the following eluents

Columns 1 and 2 (for quantitative hexosamine determination) 1 ml of 1 per cent CPC, 0 3 M NaCl, $MgCl_2$ solutions at step wise increasing concentrations from 0 25 M to 0 80 M $MgCl_2$ 2 M $MgCl_2$ and finally with 6 M HCl Columns 3, 4, 5 and 6 (for electrophoresis) 1 ml of 1 per cent CPC, 0 3 M NaCl 0 45 M $MgCl_2$ 0 50 M $MgCl_2$, 0 60 M $MgCl_2$ 0 80 M $MgCl_2$ and 2 M $MgCl_2$ All salt solutions contained 0 05 per cent CPC

The fractions from columns 1 and 2 were subjected to hexosamine determinations

Analyses on the CPC supernatant To the CPC supernatant two and a half volumes of ethanol were added and *thereafter* 4 ml of a saturated solution of sodium bisulphite in water per 100 ml solution (cf *Hjertquist & Vejlens* 1968) A fine precipitate formed and was collected after two days by centrifugation It was washed twice with distilled water to remove salts Precipitates were pooled without regard to fluoride treatment and after conversion to their sodium salts passed through a Dowex 50 (H^+) column (7 × 0 5 cm) in order to remove electron positive components The electron negative material, eluted with distilled water, was dried, weighed and analysed for its content of hexosamines (Elson and Morgan), hexose (*Dische* 1955), hexuronic acid (*Bitter & Muir* 1962) and sulphate (*Dogdson & Price* 1962) Glucosamine, galactosamine and neutral sugars were estimated by gas chromatography as their trimethylsilyl derivatives as described by *Radhakrishnamurthy et al* (1966) and *Sweeley et al* (1963) with two minor modifications The hydrolysis was carried out with 0 1 M HCl for 30 hours To separate neutral sugars and hexosamines an ion-exchange chromatography was performed on a Dowex 50 (H^+) column (7 × 0 5 cm) The neutral sugars were eluted with dis

tilled water and the hexosamines with 5 M NH_4OH solution The fractions were dried and the residue was dissolved in dimethylformamide After silylation the samples were left overnight Hydrolysis curves showed a constant maximal recovery between 28 and 33 hours

Electrophoretic characterization Electrophoretic characterization of the glycosaminoglycans was carried out on pooled fractions from two to four microcolumns as well as on precipitates from the CPC supernatant The CP glycosaminoglycan complexes were converted to their sodium salts as described by *Hjertquist & Vejlens* (1968) Electrophoresis was performed on cellulose acetate membranes as described by *Seno et al* (1970) with the modifications described by *Larsson et al* (1973) A buffer of 0 2 M calcium acetate diluted with ethylene glycol in 3 2 ratio (pH 7 25) was used The electrophoresis was run at 250 V and 2–4 mA for 4 5 to 5 hours For separation of keratan sulphate from chondroitin sulphates the fractions expected to contain keratan sulphate were also run in ammonia veronal buffer according to *Kimura & Tsurumi* (1969) and in 0 1 M HCl according to *Wessler* (1971) The migration of the material was compared with that of international standards generously provided by Dr M B Mathews, University of Chicago

Statistics

Differences were tested by means of Student s t test The precision of the determinations performed in duplicate or triplicate was calculated as the standard deviation for the single value $S_e = \sqrt{\frac{d^2}{2n}}$, where d denotes the difference between each pair of values and n the number of differences

RESULTS

The contents of hydroxyproline, total hexosamines and CPC precipitable glycosaminoglycans of cortical femoral and tibial bone are given in Tables 2 and 3 The contents are given per dry weight There were no differences between the composition of the proximal and distal part of the tibia Therefore only the values for the proximal tibia are given

From Tables 2 and 3 it can be seen that the concentration of *hydroxyproline* was significantly lower in the femur than in the tibia In both bones it had a tendency to increase with age, resulting in higher concentrations at the end of the observation period Neither ingestion nor withdrawal of fluoride had any influence upon the hydroxyproline content

The contents of *total hexosamines* and *CPC-precipitable glycosaminoglycans* (Tables 2 and 3) were similar in the two bones No alterations, either with age or fluoride treatment, could be demonstrated

Microfractionation of the CPC Precipitable Glycosaminoglycans

Analytical microfractionation of the propanol solubilized CP-glycosaminoglycan complexes was performed either on bone powder from individual animals or pooled samples from two animals The distribution among the different fractions is shown in Table 4

TABLE 2 *The Contents of Hydroxyproline Total Hexosamines and CPC Precipitable Glycosaminoglycans (GAG) in Femur and Tibia of Normal Rabbits at 11 Weeks of Age*

Bones	n	Hydroxyproline per cent	Total hexosamines per mille	CPC precipitable GAG per mille
Femur	10	1 71±0 08	1 68±0 07	0 56±0 03
Tibia	10	2 18±0 11*	1 63±0 11	0 54±0 02

The values expressed per dry weight M±S D

* $p < 0.001$ denotes the significance of differences between the bones

TABLE 3 *The Contents of Hydroxyproline, Total Hexosamines and CPC Precipitable Glycosaminoglycans (GAG) in Femur and Tibia of Normal Rabbits at 45 Weeks of Age*

Bones	n	Hydroxyproline per cent	Total hexosamines per mille	CPC precipitable GAG per mille
Femur	10	1 82±0 16	1 71±0 09	0 56±0 06
Tibia	10	2 18±0 13*	1 59±0 09	0 48±0 05

The values expressed per dry weight M±S D

* $p < 0.001$ denotes the significance of differences betwen the bones

TABLE 4 *The Mutual Distribution of Different Glycosaminoglycans in Cortical Bone of Rabbits in per cent of Total Hexosamines Eluted from the Columns*

Tibia

Group	Obs time in weeks	n	1 per cent CPC contr	1 per cent CPC exptl	0 3 M NaCl contr	0 3 M NaCl exptl	Sum of $MgCl_2$[a] contr	Sum of $MgCl_2$[a] exptl	2 M $MgCl_2$ contr	2 M $MgCl_2$ exptl	6 M HCl contr	6 M HCl exptl
I 0	7 (14)	6	19 4±8 2		7 4±0 7		70 3±5 1		1 6±0 4		1 2±0 3	
I B		5		16 9±7 0		8 4±1 7		71 7±8 3		1 6±0 4		1 4±0 6
I 0	14 (21)	8	13 1±3 7		5 7+1 5		78 2±5 3		1 4±0 4		1 6±0 3	
I B		5		12 3±2 1		6 4±1 1		77 1±2 0		2 4±0 6		1 8±0 4
II 0	24 (45)	10	18 7±3 1		7 5±1 6		70 0±3 7		1 7±0 7		2 1+0 7	
II B		7		16 5±4 9		5 8±1 2		74 1±5 7		1 8±0 4		1 9±0 4

Femur

Group	Obs time in weeks	n	1 per cent CPC contr	1 per cent CPC exptl	0 3 M NaCl contr	0 3 M NaCl exptl	Sum of $MgCl_2$[a] contr	Sum of $MgCl_2$[a] exptl	2 M $MgCl_2$ contr	2 M $MgCl_2$ exptl	6 M HCl contr	6 M HCl exptl
I 0	7 (14)	4	20 3±0 6		8 5±0 5		67 2±0 9		2 1±0 3		2 0±0 3	
I B		5		25 1±5 4		8 9±1 5		63 1±6 3		1 3±0 2		1 7±0 7
I 0	14 (21)	6	21 2±4 3		5 9±1 6		70 0±2 7		1 7±0 3		1 4±0 6	
I B		7		24 3±5 0		8 7±1 1		63 3±5 9		1 9±0 5		1 8±0 4
II 0	24 (45)	10	25 9±3 5xx		8 0±2 1		63 0±3 2xx		1 4±0 5		1 8±1 0	
II B		7		19 1±2 2***		7 6±0 9		70 3±3 0***		1 5±0 2		1 5±0 4

Groups I 0 and I B represent the period of fluoride supplement while groups II 0 and II B represent the period after withdrawal of the fluoride supplement Observe the age related increase in the 1 per cent CPC fraction in normal femur, which did not occur in the fluoride treated group
Actual age within brackets M±S D The statistical significance of differences between the groups is indicated by (*) and between observation times in the control group by (x)

xx $0\ 001 < p < 0\ 01$

*** $p < 0\ 001$

a) 0 25–0 80 M

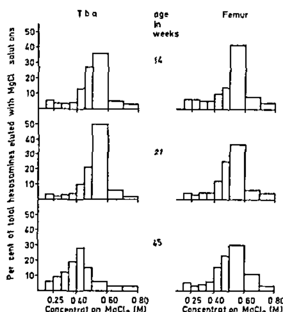


Fig 2 Solubility profiles of the CP chondroitin sulphate complexes of rabbit tibial and femoral cortical bone at different ages

The 1 per cent CPC fraction was significantly smaller ($p < 0.001$) in the tibia than in the femur On the other hand, the sum of the $MgCl_2$ fractions (0.25 M–0.8 M) was significantly higher ($p < 0.001$) in the femur

In the tibia there were no consistent changes in the mutual distribution of the different glycosaminoglycans, either with age or with fluoride treatment In the normal femur there was an age correlated increase of the CPC fraction with a corresponding decrease the sum of the MgCl fractions ($p < 0.01$) In the fluoride treated animals no such alterations were seen This resulted in a significantly ($p < 0.001$) smaller CPC fraction and a correspondingly higher sum of $MgCl_2$ fractions at the end of the observation period in the fluoride treated animals as compared with the controls

The *solubility profiles* of the CP chondroitin sulphate complexes from the tibia showed the peaks in the 0.45 M to 0.60 M MgCl fractions at the age of 14 and 21 weeks At 45 weeks of age almost all (14 out of 17) animals showed the peak in the 0.45 M MgCl fraction (Fig 2) In the femur, the majority (25 out of 39) of the profiles showed the peaks in the 0.60 M MgCl fraction without age related alterations No effect of fluoride treatment was observed

Chemical Analysis of the CPC Supernatant

In Table 5 the results of the chemical analyses of the CPC supernatant are given Approximately equal amounts of glucosamine and galactosamine were present There was a low content of sulphate No further attempt to characterize this substance was made

Electrophoretic behaviour Upon electrophoresis, no material with anodal mobility was found in the 1 per cent CPC fraction In the fraction eluted with 0.3 M NaCl traces of material with a mobility similar to that of hyaluronic acid and chondroitin 4 sulphate were occasionally observed Electrophoresis of the various fractions eluted with 0.45–0.80 M $MgCl_2$ solutions gave a distinct band with the same mobility as *chondroitin 4 sulphate*

TABLE 5 *Chemical Analyses of Pooled Precipitates of the CPC Supernatant of Cortical Femoral and Tibial Bone after Removal of Electron Positive Material*

Galactose	Hexosamines		Hexuronic acid	Non spec	Sulphate	Hexuronic acid/* galactosamine	Galactose/* glucosamine
	Glucosamine	Galactosamine					
21.6	17.8	22.3	18.8	15.4	8.8	0.78	1.21

Values in per cent of dry weight
* Expressed as molar ratio with the hexosamine as 1.00

Fig 3 Electrophoresis in 0 2 M calcium acetate pH 7 25 on cellulose acetate membrane of fractions eluted with d fferent salt concentrations from CPC microcolumns Samples from left to right 1 Standard CSC, 2 CSA 3 0 45 M $MgCl_2$ 4 0 50 M $MgCl_2$ 5 0 60 M $MgCl_2$, 6 0 80 M $MgCl_2$ All MgCl fractions show identical anodal mobility similar to the CSA standard

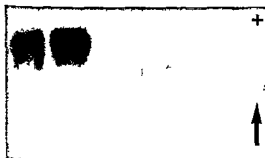


Fig 4 Electrophoresis in ammonia veronal buffer, pH 8 6 on cellulose acetate membrane Samples from left to right 1 standard CSA 2 CSC, 3 CPC supernatant, 4 standard KSl 5 CPC super natant, 6 KSl In the CPC supernatants material with anodal mobility similar to the keratan sul phate is present as well as material with slower and faster mobility

(CSA standard) in all samples studied (Fig 3) No difference in the anodal mobility was found between materials eluted with solutions of increasing molarity Traces of material with a mobility like that of chondroitin 4-sulphate were occasionally found in the 2 M MgCl and 6 M HCl fractions

Electrophoresis of the precipitates from the CPC supernatants showed in acetate buffer, ample presence of material with anodal mobility but with a high degree of heterogeneity In veronal buffer material was demonstrated with mobility corresponding to that of keratan sulphate (KS 2 standard) and chondroitin [illegible] standard) (Fig 4) Also in 0 1 M hydrochloric acid material with a mobility corresponding to keratan sulphate (KS 1 standard) was demonstrable

No differences in electrophoretic behaviour were found either with age or fluoride treatment

DISCUSSION

For the analyses of the glycosaminoglycans in cortical bone from the tibia and femur of growing and adult rabbits, the CPC precipitation method was used as described and discussed in detail by *Hjertquist & Vejlens* (1968) Since the analyses were performed on samples from individual animals only small amounts of material were available, therefore further characterization of the fractions had to be attempted by electrophoresis The results showed the presence of chondroitin-4 sulphate as the main glycosaminoglycan This is in agreement with earlier chemical studies in rabbits (*Vejlens* 1971 a), dogs (*Hjertquist & Vejlens* 1968), oxen (*Meyer et al* 1956) and rats (*Larsson & Vejlens* 1969) and electrophoretic studies in rabbits by *Burckard et al* (1966) However, by electrophoresis, *Iwata & Urist* (1972) also found chondroitin 6 sulphate Traces of a glycosaminoglycan with electrophoretic mobility similar to that of hyaluronic acid were also present This is in agreement with the findings obtained by *Burckard et al* (1966) and compatible with the detection of a glucosaminoglycan by *Hjertquist & Vejlens* (1968) in the 0 3 N NaCl fraction

In contrast to the findings obtained by *Hjertquist & Vejlens* (1968), minor amounts of hexosamine containing material were precipitated from the CPC supernatant in our material Its presence in the CPC supernatant suggests that it may be keratan sulphate (cf *Scott* 1960, *Antonopoulos et al* 1964) Chemical analysis showed a concentration of 40 per cent of hexosamines per dry weight and approximately equal amounts of galactosamine and glucosamine The sulphate content was low Further attempts to character-

ize this substance were considered to be beyond the scope of this study These chemical data suggested the presence of both keratan sulphate and chondroitin sulphate of low molecular weight and/or sulphation Glycopeptides containing amino sugars and shown to be present in bone (*Herring* 1968) were eliminated by a chromatographic step and cannot have interfered to any noteworthy degree Also the low ratio of hexose to hexosamine suggests the absence of any major amount of glycopeptides Electrophoreses showed heterogeneity of the material, but part of it had a mobility compatible with that of keratan sulphate Keratan sulphate was reported to be present in rabbit diaphyseal bone studied by electrophoresis by *Iwata* & *Urist* (1972)

The hydroxyproline concentration was not affected by the fluoride ingestion Thus the suggestion by *Peck et al* (1965) that fluoride reduced total bone collagen was not supported

The concentration of hydroxyproline was at all ages lower in the femur than in the tibia which is in accordance with collagen values given by *Eastoe* (1961) for rabbit diaphyses of femur and tibia The biological significance of this finding, however, is unknown

Age related changes were small The solubility profiles of the CP chondroitin sulphate complexes of the tibia showed a shift of the peak from the 0 60 M $MgCl_2$ fraction in the growing animals to the 0 45 M $MgCl_2$ fraction in the adult rabbits eg to chondroitin sulphate of lower molecular weight and/or charge density No such shift was seen in the femur The mutual distribution of different glycosaminoglycans was unaltered in the tibia In the femur there was a relative increase of the CPC fraction and a corresponding decrease of the sum of the MgCl fractions (chondroitin sulphate) with increasing age

The cortical bone of tibia and femur of growing rabbits given fluoride (10 mg/kg body weight/day) showed an accumulation of fluoride in the bone mineral increasing to about twentyfold that in the controls during the growth period (*Rosenquist* 1973 a) During the same period, an enormous, mainly periosteal, new bone formation occurred in the diaphysis of the tibia This newly formed bone had a morphology clearly different from that of normal cortical bone, but the degree of mineralization appeared to be normal as judged from microradiographs (*Rosenquist* 1973 b) This new bone formation was accompanied by increased bone resorption The bone tissue formed during the period of fluoride ingestion constituted a considerable part of the total bone present at the end of the ingestion and growth period In view of this, it may be stated that fluoride ingestion and high fluoride contents in the bone mineral do not affect the ability of the osteoblastic cells to synthesize normal amounts of glycosaminoglycans with chemical and physical properties apparently similar to those found in the controls

The only effect demonstrable in the fluoride treated animals was the lack of the age related alterations in the mutual distribution of different glycosaminoglycans in the femur This occurred in spite of the small morphological alterations in this bone compared with the tibia where excessive bone formation gave no chemical alterations

Thus, in this investigation on cortical diaphyseal bone it has not been possible to demonstrate either quantitative or qualitative changes in acid glycosaminoglycans in spite of pronounced bone resorption as well as new bone formation These observations are in general agreement with the findings obtained by *Vejlens* (1971 a and b) under various experimental conditions and in certain human bone diseases characterized by the presence of excessive amounts of cortical bone, ie osteopetrosis (*Vejlens* 1972) and Camurati Engelmann s disease (*Lemperg* unpublished observations)

At the time of this investigation no method for small scale analysis of proteoglycan complexes was available Possibly, future investigations of the proteoglycans may give more insight in to the role of these

polysaccharides in the process of bone formation and maturation

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Financial support was given by *Reservationsanslaget för främjande av ograduerade forskare, Stiftelsen Sigurd och Elsa Goljes Minne* and the *Swedish Medical Research Council* (24 X 3925 and 17 X 138)

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INHIBITION OF LYMPHOCYTE PROLIFERATION *IN VITRO* BY A SPLENIC FACTOR

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A splenic factor was prepared from a saline extract of calf spleen by centrifugation at high speed, heat precipitation and aceton precipitation The factor had an inhibitory effect on the DNA synthesis of lymph node cells and thymus cells *in vitro* The possibility that the factor is a T cell chalone is discussed

In a previous investigation a saline extract of calf spleen has been shown to inhibit the thymic release of lymphocytes into the blood of guinea pigs (*Ernstrom* 1972) In the present paper a splenic extract is shown to inhibit DNA synthesis in lymphocytes Based on an *in vitro* test the inhibitory splenic factor has been partly purified and the effect of the factor further analysed

MATERIAL AND METHODS

Animals Male and female guinea pigs weighing 250–300 g were used as cell donors for the *in vitro* experiments

Chemicals Hank's solution and PBS (Dulbecco) were obtained from the State Bacteriological Lab (Stockholm, Sweden) and RPMI medium 1640 with glutamine, penicilline and streptomycin from Flow Labs The scintillation fluid used consisted of 5 g PPO and 0 2 g POPOP in 1000 ml Toluene

Fractionation procedure Frozen calf spleen, obtained from a local abattoir was homogenized in 0 9 per cent saline with a tissue saline ratio of 1 3 The homogenization was performed in a Waring Blendor at 0–5° C All the f llo ng isolation procedures were carried c [illegible] in the cold The homogenate was centrifuged [illegible] 1200 g in a MSE refrigerated centrifuge for [illegible] The supernatant fluid was then centrifuged [illegible] 46 000 g for 2 h The supernatant fluid was subje [illegible] to heat at 80° C for 15 min and then cooled in an ice bath A large, brown, voluminous precipitate formed and was removed by centrifugation at 50,000 g for 15 min To the clear supernatant solution 10 volumes of cold (–20° C) acetone was slowly added during constant stirring This procedure was carried out in the tubes used for centrifugation The precipitate was collected by centrifugation at 4,000 g for 15 min and then broken up with a glass rod and recentrifugated in 3 volumes of acetone The supernatant was again removed and the precipitate was dried under reduced pressure The powder obtained was solubilized to about 80 per cent when ground with a glass rod and extracted with 0 1 M sodium phosphate buffer (pH 7 2) The small insoluble residue was removed by centrifugation The solution was lyophilized and stored in a freezer

A control extract of calf liver was prepared in the same way In one experiment our extracts were compared with an extract of calf thymus, called thymosin, prepared by Dr Goldstein (*Goldstein et al* 1972)

Before use of our extracts, determination of the protein content was performed according to *Lowry et al* (1951)

Cell culture Guinea pigs were anaesthetized with sodium mebumal and were used as cell donors The thymus or cervical and mesenterial lymph nodes or spleen was removed aseptically and placed in cold Hank s BSS and PBS (Dulbecco) The cells were teased gently and passed through a sterile net and then collected by centrifugation The supernatant fluid was removed and the cells were washed with buffer solution After centrifugation the supernatant fluid was removed and the washed cells

Received 4 ii 74 Accepted 4 ii 74

were suspended in a small volume of medium RPMI 1640, containing l-glutamine (200 mM/ml), streptomycin (100 IU/ml) and penicillin (100 IU/ml) The suspension was adjusted to a concentration of $1-10 \times 10^6$ lymphoid cells per ml Of this suspension 1 ml was transferred to each of a number of Falcon plastic tissue culture tubes (12×75 mm) The tubes were incubated at 37° C in 10 per cent CO_2 in air The spleen or liver extract dissolved in 0 2 ml saline was added to the cultures 30 min after start of incubation

Thymidine uptake ^{3}H thymidine, 2 μCi (specific radioactivity 5 Ci/mM, Radiochemical Centre, Amersham) in 0 05 ml saline was added to each culture 0 5 or 1 h before harvest of the cultured cells The incubation was interrupted by putting the tubes in an ice bath The content in each tube was poured on a membrane filter (Sartorius, SM 11304) in a "Manifold' multiple sample collector (Millipore Corp) The cells on the filters were washed twice with 10 ml saline and once with 10 ml 5 per cent trichloro acetic acid The filters were then put into scintillation vials and dried at 60° C for 30 min Finally, 10 ml of the toluene scintillation fluid was added to each vial and the samples were counted in a liquid scintillation spectrometer Results were expressed as cpm/culture (mean value of samples from three cultures and in some experiments from five cultures) Background activity was neglible and was not subtracted The range was as a rule less than 10 per cent of the mean Selected examples from typical experiments are seen in Fig 1–4

Experiments The following types of experiments were performed

(1) The cultured cells were taken from different organs (thymus, lymph node, spleen) and exposed to the different extracts

(2) The kinetics of the ^{3}H thymidine uptake in short time cultures exposed to the different extracts was determined

(3) Dose response experiments for the different extracts were done In most experiments, the doses were adjusted to give the same amount of protein in the thymus liver and spleen fractions used

(4) The viability of the cultured cells was determined by trypan blue exclusion test at the end of the incubation

RESULTS

When the growth of cultured lymphoid cells from different sources was compared, the incorporation of ^{3}H-thymidine in cultures of thymic cells was greater than in cultures of lymph node cells and spleen cells Our splenic extract was added to the cultures and the effect of the extract on the incorporation of

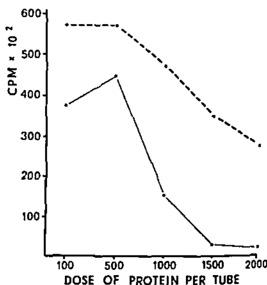


Fig 1 Influence of different doses of spleen and liver extract on the growth of thymic cells *in vitro* Each culture contained $5 6 \times 10^6$ cells per ml The doses of extract are given as μg protein per tube Cells were harvested after 5 h in culture ^{3}H TdR was added 1 h before harvest Each point represents the mean of triplicate cultures
——— spleen extract, – – – – – liver extract

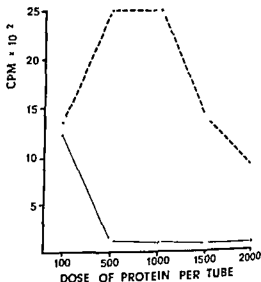


Fig 2 Influence of different doses of spleen and liver extract on the growth of lymph node cells *in vitro* Each culture contained $1 2 \times 10^6$ cells per ml The doses of extract are given as μg protein per tube Cells were harvested after 5 h in culture ^{3}H TdR was added 1 h before harvest Each point represents the mean of triplicate cultures
——— spleen extract, – – – – liver extract

^{3}H thymidine in the cultured cell was studied The results showed that the splenic extract inhibited the growth of thymic cells and lymph node cells but not that of splenic cells Control cultures were exposed to saline or to an identically prepared extract of calf liver (Fig 1 and 2)

Dose response experiments were performed with our aceton precipitated and lyophilized material A marked inhibition of the proliferation of lymph node cells was obtained with 500 μg of the splenic extract while 1500 μg of the same extract per culture was needed to give a similar inhibition of thymic cells

The importance of the time during which the cultures were exposed to the splenic extract was investigated A decreased incorporation of ^{3}H thymidine in thymic cells was found after 5 h exposition and a still more pronounced decrease after 6 and 7 h (Table 1) Experiments with shorter incubation periods (0 5–3 h) for cultures with thymic cells did not result in any inhibition of the proliferation by the splenic extract (Table 2) Similar results were obtained with lymph node cells, though increasing inhibition by the splenic extract was demonstrated as early as from 2 h and onwards (Fig 4)

TABLE 1 *Time Study of the Effect of Saline, Spleen Extract and Liver Extract on the Incorporation of ^{3}H Thymidine in Thymic Cells* in vitro

Culture time	Saline cpm/tube	Spleen extract cpm/tube	Liver extract cpm/tube
3 h	43256	67025	43352
4 h	38796	43196	30037
5 h	37771	12272	23278
6 h	43358	1238	20234
7 h	37278	192	17346
8 h	30002	182	14087

8 mg of the crude extract in 0 2 ml saline was added to each tube at the start of the experiments ^{3}H thymidine was added 30 min before harvest The figures in the three columns represent three different experiments and are not directly comparable Each figure represents the mean cpm of 5 cultures Inhibition of DNA synthesis was demonstrated by the spleen extract

TABLE 2 *Time Study of the Effect of Saline, Spleen Extract and Liver Extract on the Incorporation of ^{3}H Thymidine in Thymic Cells* in vitro

Culture time	Saline cpm/tube	Spleen extract cpm/tube	Liver extract cpm/tube
0 5 h	7105	15268	47137
1 h	7548	23157	52228
1 5 h	6727	24058	51242
2 h	7730	20079	55133
2 5 h	7989	18351	47642
3 h	9587	18115	37243

8 mg of the crude extract in 0 2 ml saline was added to each tube at the start of the experiments ^{3}H thymidine was added 1 h before harvest The figures in the three columns represents three different experiments and are not directly comparable Each figure represents the mean cpm of 5 cultures No inhibition of DNA synthesis was demonstrated during the first 3 h of culture

The effect of our splenic extract was also compared to that of a thymic extract, called thymosin (see *Goldstein et al* 1972) In contrast to the marked inhibition found if the splenic extract was used, the cultures exposed to thymosin showed high incorporation of ^{3}H thymidine with maximal values using 1000 μg and 1500 μg thymosin, indicating that thymosin stimulates lymphocyte proliferation (Fig 3)

The splenic extract obtained after aceton precipitation and lyophilization contained 20–30 per cent protein and had maximal absorbtion at 260 nm The lyophilized extract could be stored for at least 6 months at –20° C without loss of the inhibitory effect on lymphocyte proliferation Preliminary results obtained by separation on Sephadex G50 indicates that the inhibitory factor has a mol weight less than 30,000

The viability of the cultured lymphoid cells was studied in separate experiments The viability was more than 99 per cent in most cultures exposed to the spleen or liver extracts Only exceptionally a viability of 80–99 per cent was found, but this lower viability was seen in a few cultures exposed to spleen and liver extracts as well as to saline Thus no cytotoxic effect of the extracts on the

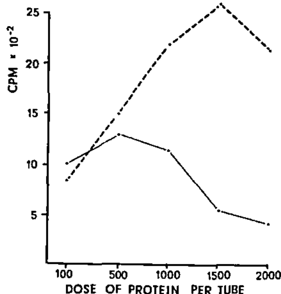


Fig 3 Influence of different doses of spleen and thymus extract on the growth of lymph node cells *in vitro* Each culture contained 1 7 × 10^6 cells per ml The doses of extract are given as µg protein per tube Cells were harvested after 5 h in culture ^{3}H TdR was added 1 h before harvest Each point represents the mean of triplicate cultures ——— spleen extract, – – – – – – thymic extract

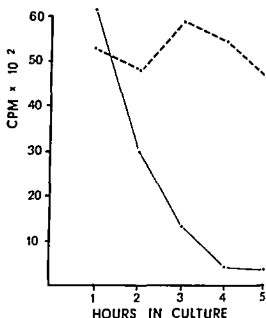


Fig 4 Time study of the inhibitory effect of the spleen extract on the proliferation of lymph node cells *in vitro* Each culture contained 1 5 × 10^6 cells per ml 800 µg protein per tube ^{3}H TdR was added 1 h before harvest Each point is the mean of triplicate cultures ——— spleen extract, liver extract

cultured cells could be demonstrated by this dye exclusion test

DISCUSSION

Growth inhibitory factors specific for different tissues but not species specific have been postulated by *Bullough* (1962) and are known as chalones They are supposed to regulate the growth of liver, kidney, skin, granulocytes etc (*Saetren* 1956, *Echave Llanos* 1967, *Bullough & Laurence* 1968, *Rytomaa & Kiviniemi* 1968, *Simnett & Chopra* 1969) Chalones for lymphocytes have also been reported Thus, *Moorhead et al* (1969) prepared an extract from pig lymph nodes, *Garcia-Giralt et al* (1970) from bovine spleen and *Houck et al* (1971) from rat lymph nodes and spleen The extracts were found to inhibit DNA synthesis in lymphoid cells without affecting the viability of the cells The active component of the extracts was reported to be a macromolecule with a mass of 30,000 to 75,000 daltons, it was thermolabile and destroyed by trypsin and not stabile on long-term storage (*Jones et al* 1970, *LaSalvia et al* 1970, *Houck et al* 1971) The inhibitory material was reported to lack discrimination between T and B cells (*Houck* 1973)

Different factors have also been isolated from the thymus, some of which are stimulating and others inhibiting DNA synthesis in lymphocytes *in vitro* (for review see *Luckey* 1973) A lymphocyte specific inhibitory factor from calf thymus has been prepared by *Kiger* (1971) This material interfered with the rejection of skin allografts in mice and inhibited the GVHR as well as the transformation of lymphocytes stimulated by PHA (*Kiger et al* 1972)

In the present paper, a splenic factor characterized by an inhibitory effect on the DNA synthesis of lymph node cells and thymic cells *in vitro* has been partly purified by centrifugation at high speed, heat precipitation at

80° C and by acetone precipitation Our *in vitro* experiments indicate a certain difference between the sensitivity of lymph node cells and thymus cells Thus lymph node cells are sensitive to a lower dose of the inhibitory material and seem to be inhibited earlier after addition of the factor to the culture Preliminary studies using separation on Sephadex G50 indicate that our factor has a molecular weight less than 30,000 The active component appears to be thermo stabile and can be stored at -20° C for at least 6 months It is evident that our splenic factor is not identical with the bovine splenic factor of *Garcia Giralt* The marked inhibitory effect of our splenic factor on the proliferation of lymph node cells and thymus cells *in vitro* and on the release of lymphocytes from the thymus *in vivo* (*Ernström* 1972) raises the question whether the factor may be specific for T cells as is proposed for the factor of *Kiger et al* (1972) However, before our factor can be regarded as a lymphocyte chalone, the specificity as regards the target tissue must be further analysed and the effect in vivo further investigated

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This investigation was supported by the *Swedish Cancer Society* (project No 511 B72 02X) and by the *Swedish Medical Research Council* (project No B74 12X-4206-01)

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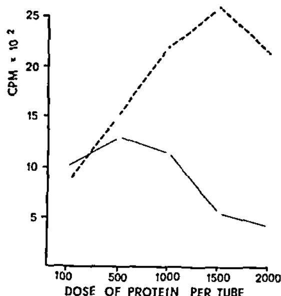


Fig 3 Influence of different doses of spleen and thymus extract on the growth of lymph node cells *in vitro* Each culture contained 1 7 × 10⁶ cells per ml The doses of extract are given as µg protein per tube Cells were harvested after 5 h in culture ³H TdR was added 1 h before harvest Each point represents the mean of triplicate cultures ——— spleen extract, --- --- thymic extract

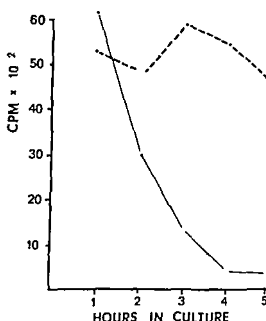


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In the present paper, a splenic factor characterized by an inhibitory effect on the DNA synthesis of lymph node cells and thymic cells *in vitro* has been partly purified by centrifugation at high speed, heat precipitation at

MATERIAL AND METHODS

The material consists of four groups of female Sprague-Dawley rats weighing between 180 and 200 g at the beginning of the experiments The rats were kept on tap water *ad libitum* and laboratory chow containing 0 4 per cent sodium chloride The blood pressures were assessed by the tail pletysmo graphic method with the rats under brief ether anesthesia Blood pressure measurements were made on three different occasions before the experiments started and at regular intervals thereafter Hyper tension was considered to have developed when the blood pressure became stabilized above 140 mm Hg, provided that this included a rise of mini mum 15 per cent of the original blood pressure The initial blood pressure ranged between 85 and 125 mm Hg

Group I Unilateral renal artery stenosis was produced in rats by the application of a 0 15 mm wide silver clip on the left renal artery of the anaesthetized rat This group consists of fourteen rats in which hypertension developed following the operation The rats were killed within the first hypertensive week This means that they were killed 3 5 weeks following the operation, since the latent phase from operation to the development of hyper tension ranged between 2 and 4 weeks

Group II In this group of twenty five rats the left renal artery of the anaesthetized rat was ex posed and all periarterial and adventitial tissue carefully removed throughout the entire length of the artery The rats were killed 3–5 weeks after the operation to correspond to the rats of group I

Group III In this group of fifteen rats the denervation procedure described for the rats of group II was combined with the application of a 0 15 mm wide silver clip on the left renal artery The rats were killed at intervals corresponding to those for the rats in groups I and II

Group IV In this group of thirteen rats the denervation procedure was followed three weeks later by the application of a 0 15 mm wide silver clip on the left renal artery The animals were killed 3 5 weeks after the second operation to correspond to the rats of groups I and III

After the animals were killed the left and right kidneys were quickly removed and pieces taken and processed for demonstration of biogenic monoamines according to the histochemical fluorescence method (*Falck et al* 1962 *Corrodi & Jonsson* 1967 and *Fuxe et al* 1970) Some pieces from each kidney were processed directly whereas other pieces first were incubated in α methyl norepinephrine of various concentrations (5×10^{-4}M and 5×10^{-5}M) as previously described (*Unge et al* 1973)

After processing all kidney pieces were vacuum embedded in paraffin and 3 μ thick sections cut, mounted in Entellan (Merck) to which xylol was added, and examined in the fluorescence mi croscope

RESULTS

Blood Pressure Reaction

The number of hypertensive animals in the various groups is presented in Table 1 By definition all rats in group I were hyper tensive In group II two rats were hypertensive at death with a blood pressure level of 170 mm Hg in both cases In group III six rats developed hypertension with a blood pressure between 160 and 180 mm Hg at death In group IV three rats became hypertensive with blood pressure levels of 150, 150 and 160 mm Hg at death One more rat in this group was possibly hypertensive with a blood pressure elevation from 90 to 140 mm Hg

Nerve Reaction

In all groups of rats the right kidneys showed an entirely normal adrenergic nerve pattern with yellowish-green fluorescence of varicose fibers distributed along the intrarenal arterial and arteriolar system (*Ljungqvist & Wågermark* 1970) No differences were recorded between the incubated and non in cubated specimens

For the left kidney differences in the adre nergic nerve system were noted between the various groups of rats and between incubated and non incubated specimens In some specimens fluorescent nerve fibers were more sparse than normally, particularly around the finer intrarenal arteries In such instances the fluorescence usually also had a lowered intensity These nerve alterations are referred to as a "reduced" nerve pattern In other specimens either no fluorescent fibers were found or occasional weakly fluorescent fibers were seen along the larger intrarenal arteries This change is referred to as an "abolished" nerve pattern The distribution of the nerve patterns in the incubated and non-incubated specimens from the various groups of rats is summarized in Table 1

TABLE 1 *The Distribution of the Nerve Patterns in Specimens from the Left, Operated Kidney not Incubated and Incubated in a methyl norepinephrine*

Group	No of rats	H t	Not incub			Incub		
			N	R	O	N	R	O
I	14	14	4	0	10	10	2	2
II	25	2	3	3	19	8	0	17
III	15	6	3	0	12	3	0	12
IV	13	3	0	0	13	1	0	12

Group I Left renal artery stenosis with hypertension <1 week
Group II Left renal denervation
Group III Left renal denervation + renal artery stenosis
Group IV Left renal denervation + renal artery stenosis 3 weeks later
N = normal pattern R = reduced pattern O abolished pattern H t = No of hypertensive rats

Relation of Nerve Reaction to Blood Pressure

For the renal hypertensive rats (group I) it can be seen that the nerve pattern was abolished in the non incubated specimens from ten stenosed kidneys whereas in eight of these kidneys a reduced or normal nerve pattern appeared in the incubated specimens (Fig 1)

For the rats in which denervation only was performed (group II) no significant difference in the nerve patterns were noted between the incubated and non incubated materials Two rats in this group had been hypertensive for 2 weeks when killed and in these the nerve pattern in the left kidney was abolished both in the non incubated and the incubated specimens Consequently, all eight rats in which a normal nerve pattern was seen after incubation were normotensive

For the rats in which left renal artery stenosis and left renal denervation was performed simultaneously (group III) the nerve findings were identical in the incubated and non incubated materials Persistent hypertension developed in one of the rats with normal left kidney nerve pattern and in five of the twelve rats in which the nerve pattern was abolished In the hypertensive rats the blood pressure elevation had been present for about 3 weeks when the rats were killed

For the rats in which renal denervation was followed by renal artery stenosis 3 weeks later (group IV) all left kidneys had an abolished pattern in the non incubated material In the incubated specimens, however numerous nerve fibers with a normal distribution were seen in one kidney This kidney derived



Fig 1 Fluorescence micrograph from left (stenosed) kidney of early hypertensive rat (hypertension for less than 1 week) The kidney specimen was incubated in norepinephrine before the histochemical reaction Varicose fluorescent fibers are seen along an intrarenal arteriole In non incubated specimens from this kidney no fluorescent fibers were seen × 300

from a rat in which hypertension had been present for 3 weeks. The other two hypertensive rats in this group had an abolished nerve pattern both in the incubated and non-incubated specimens, and these rats had been hypertensive for 1 and 2 weeks respectively.

DISCUSSION

Numerous experimental data have been obtained over the last few years in which the importance of the renal sympathetic nervous system for the release of renin from the kidney has been demonstrated. *Vander* (1965) showed that electrical stimulation of the renal nerves will result in increase in plasma renin activity and *Johnson et al.* (1971) recorded an increased release of renin during norepinephrine infusions. These effects may be explained as the result of a direct action by the adrenergic transmittor substance on the renin-producing juxtaglomerular cells, since these cells are surrounded by adrenergic nerve terminals (*Wågermark et al.* 1968).

In hypertension secondary to renal artery stenosis the blood pressure elevation is known to be the result of an increased release of renin from the juxtaglomerular cells of the stenosed kidney. The sequence of events leading to the increase in renin release is not fully understood. Recently a depletion of the adrenergic transmitter in the nerve terminals related to the juxtaglomerular cells of the stenosed kidney was demonstrated during the developmental phase of the hypertension (*Ljungqvist & Ungerstedt* 1972). In the absence of a similar reaction in the stenosed kidney of animals in which hypertension did not develop it was suggested that the depletion was the result of an increased release of the substance from the nerve terminals with a consequent stimulation of the juxtaglomerular cells and increase in the release of renin. The possibility that [illegible] depletion of the transmitter was the resu[illegible] of a denervation caused by the surgical [illegible]dures could however not be entirely [illegible]ut.

In the present [illegible]tigation a deple[illegible] of the adrenergic tr[illegible]itter in the [illegible]osed kidney from most animals with early hypertension was again demonstrated (group I). It was also shown that in the vast majority of these kidneys the nerve terminals were capable of taking up norepinephrine *in vitro*, suggesting that they were not damaged by the surgical intervention. The results therefore lend further support to the view that the depletion of the transmitter is due to an increased release of the substance, and this may be of importance for the blood pressure elevation by promoting the release of renin.

It has previously been shown that a normal nerve pattern will reappear in the stenosed kidney after the initial phase of the blood pressure elevation (*Ljungqvist & Ungerstedt* 1972). The existence of a normal nerve pattern in the non incubated material from the stenosed kidney in four "early" hypertensive rats therefore suggests that these rats had, in fact, experienced a prolonged blood pressure elevation although not assessable by the methods used.

The fact that hypertension developed in a significant number of animals in which the stenosed kidney was completely denervated indicates that renal adrenergic nerve activity is not the basic event in the stimulation of the renin release mechanism which occurs secondary to renal artery stenosis. This is in good agrement with previous observations that the renal sympathetic nerves play a secondary and moderatory role in the release of renin under various patho-physiological conditions, rather than being involved in the primary pathogenetic events (*Vander & Luciano* 1967, *Nomura et al.* 1972).

The blood pressure elevation recorded in two animals in which only the denervation operation was performed probably resulted from a reaction in the traumatically injured periarterial tissue with the secondary development of a renal artery stenosis. Such a reaction would be reasonable to except in a limited number of animals subjected to this type of denervation operation.

The demonstration of a normal nerve pattern in eight rats in which only denervation operation was performed simply indicates that

either was the denervation incomplete or had a re-innervation occurred This emphasizes the importance of a histochemical control of the effect of a denervation operation on an organ before conclusions can be drawn on the basis of experimental data obtained

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This study was supported by the *Swedish Medical Research Council* (Project No B72-12X-716-07A) and the *Research Funds of Karolinska Institutet*

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Acta path microbiol scand Sect A, 82, 455–458, 1974

ON THE INFLUENCE OF EXOGENEOUS CYCLIC AMP (3', 5'-ADENOSINE MONOPHOSPHATE) ON AMYLOID FORMATION IN CASEIN-TREATED C_3H MICE

P EBBESEN

Department of Tumor Virus Research, Institute of Medical Microbiology
22 Juliane Maries Vej DK 2100 Copenhagen Ø Denmark

Amyloid formation in caseinated C_3H mice is enhanced by the administration of dibutyryl cyclic AMP (3', 5, adenosine monophosphate), by the phosphodiesterase inhibitor theophylline and by combination of these two compounds One beta receptor stimulator, soterenol, also enhanced amyloid development, but several other α and β receptor active substances tested were without effect in the dose used It is hypothesized that a sustained elevation of intracellular cyclic AMP level can make cells produce amyloid

Amyloid formation in mice during casein treatment is accelerated by simultaneous treatment with either of the polycations DEAE dextran and Polybrene (*Ebbesen* 1972 a) These polycations facilitate several membrane functions (*Toyoshima & Vogt* 1969, *Ebbesen* 1972b), consequently we have tested for influence on amyloidogenesis of "the second membrane messenger', i e cyclic AMP (*Robinson et al* 1968)

MATERIALS AND METHODS

Animals used in these experiments were two-month-old inbred C_3H female *mice* fed on mouse pellets and water ad libitum *Casein* was administered with 0 5 ml of a 5 per cent solution in 0 25 per cent NaOH subcutaneously 6 times a week for 5 weeks. 30 minutes after each casein inoculation the pharmaca were injected intraperitoneally with the below doses Each pharmacon was administered to 10 mice also receiving casein and to 5 mice receiving saline

Received 1.xii 73 Accepted 5 ii 74

Minimum Essential Medium (Eagle) with Hanks' Balanced Salt Solution pH 7 2, was used as diluent for the pharmacons used There were

Tetra sodium pyrophosphate $Na_4S_2O_6$

N° 2_1O dibutyryl A 3 5 MP (*Cyclic AMP*)
Cat No 15205 NAAT

Theophylline
Pharmacopea Danica

Noradrenalin 0 1 per cent
Pharmacopea Danica

Dibenamin HCl
KPK Laboratories Inc
Plainview N Y Hollywood, California

Isoprenalini sulfatis 0 1 per cent
Pharmacopea Danica

Soterenol
Mead Johnson & Co, Evansville 21, Indiana, U S A

Salbutamol
2 t butylamino-1-(4 hydroxy 3 hydroxymethyl) Phenylethanol
Allen & Hanburys Ltd, Ware, London, England

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Acta path. microbiol scand Sect A, 82, 45> 4] ‹

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Tetra sodium pyrophos[illegible]

N^6 2,O-dibutyryl A 3 5 Cat No 15205 NAA[illegible]

Theophylline Pharmacopea Danica

Noradrenalin 0 1 per c[illegible] Pharmacopea Dan[illegible]

Dibenamin HCl KPK Laboratories [illegible] Plainview N Y [illegible]

Isoprenaline sulfa[illegible] Pharmacopea D[illegible]

Soterenol Mead Johnson [illegible] U S A

Salbutamol 2 t butylamino-[illegible] Phenylethanol Allen & Hanb[illegible]

Received 1 xii 73 Accepted 5 ii 74

Practolol INN (Eraldin)
Emperial Chemical Industries Ltd
Alderley Park, Macclesfield, Cheshire, England

Propranololi chloridum (Inderal)
Imperial Chemical Industries Ltd
Alderley Park, Macclesfield, Creshire, England

Adrenalin 0 1 per cent
Pharmacopea Danica

All animals were killed on day 35 Lung, liver, spleen, kidney, mesenteric lymph node, peripheral lymph nodes cor, thymus and thyroid gland were taken for *microscopy* and stained with periodic acid Schiff (PAS) and alcaline Congo red Amyloid was identified by its birefringence with Congo red under crossed polars The degree of spleen amyloidosis was determined on PAS stained sections (*Christensen* & *Hjort* 1959), a continuous ring of amyloid around spleen follicles being rated as grade 3

RESULTS

All caseinated mice developed amyloid around the spleen follicles If present in grade 4 or 5, the liver also showed amyloid around vessels and in sinusoids Other organs were free of amyloid detectable by light microscopy

Cyclic AMP and theophylline both significantly enhanced the amyloid development if administered with 0 5 mg and 25 µg per day respectively These observations were certified by a repetition of this part of the experiment (Table 1) Also a combination of cyclic AMP and theophylline promoted amyloid formation Of the α and β receptor active substances tested, only soterenol had a detectable effect, soterenol being a promotor of amyloid formation

Animals receiving pharmacon but no casein never developed amyloid A few clusters of perifollicular diastase-PAS positive cells were present in the spleens of most mice having received cyclic AMP plus theophylline *but in no other mice*

DISCUSSION

The enhancing effect of synthetic exogeneous cyclic AMP which enters cells (*Falbriard et al* 1967) and of theophylline which inhibits

TABLE 1 *Spleen Amyloid in C_3H Mice given 30 Injections of Casein and Pharmacon (6 Injections a Week)*

No of mice*	Pharmacon	Dose per treatment	Type of action	Spleen amyloid Mean	Range	P
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20	Cyclic AMP	0 5 mg	second messenger	4 1	3 5	<0 01
10	Theophylline	2 5 µg	Phosphodiesterase inhibitor	3 1	2–4	
20		25 µg		4 2	3–5	<0 01
10	c AMP Theophylline	0 5 25		4 5	3 5	<0 01
20	Noradrenalin	25 µg	α stimulator	2 4	2–4	
10	Dibenamin	250 µg	α inhibitor	2 8	2–4	
20	Isoprenalin	250 µg	β stimulator	2 9	2–4	
10	Sorterenol	25 µg	β stimulator	3 8	3 5	
10	Salbutamol	25 µg	β stimulator	3 5	3–4	
20	Practolol	25 µg	β inhibitor	2 3	0–3	
10	Propranolol	25 µg	β inhibitor	2 6	2–4	
10	Adrenalin	25 µg	α + β stimulator	3 2	2–4	

* Each pharmacon in addition was administered to 5 mice treated with saline instead of casein No amyloid occurred in these animals

degradation of endogeneous cyclic AMP (*Butcher & Sutherland* 1962), point to the involvement of cyclic AMP in amyloidogenesis Stimulation of α or β-adrenergic receptors may suppress, respectively enhance, the intracellular c-AMP level (*Robinson et al* 1967) Although one β-stimulator enhanced amyloid formation, our work leaves the possibility of influencing amyloid development by these α and β receptor-active pharmacons unresolved

Cyclic AMP is "the second messenger" for cell membranes being stimulated with certain hormones (*Sutherland* 1972) and is also participating in membrane reactions to at least some antigens (*Ishizuka et al* 1970, *Weissman et al* 1971, *Bourne et al* 1972), cyclic AMP furthermore seems to be involved in phagocytosis (*Kowalski et al* 1972), in membrane reaction to antibody-complement (immune cytolysis) (*Ebbesen & Arnung* 1973), and in growth control (*Johnson & Pastan* 1972) Depending on which specific membrane receptor is stimulated, the non-specific action of cyclic AMP by difference in site of release will influence different metabolic pathways (*Pastan & Perlman* 1971)

The systemic administration of cyclic AMP-theophylline used here leaves open the possibility that the observed influence on the amyloid-producing tissue is secondary to a cyclic AMP effect on other (hormone producing?) (*Posner* 1971) organs Supported by prior evidence (*Ebbesen* 1972 a) of the importance of membrane functions for secondary amyloidosis and by the enhanced incidence of pancreatic islet amyloid in diabetics (*Lacy* 1967) where an elevated level of cyclic AMP occurs (*Exton & Park* 1968), we consider a direct effect of cyclic AMP on the amyloid-producing cells more likely

It is therefore hypothesized that external membrane stimuli (*Teilum* 1956) and endogeneous alterations causing a sustained elevation of the intracellular cyclic AMP level may lead to amyloid formation Such a concept would allow both immune and non immune induction of amyloid (*Pearse et al* 1972), and fit with the urrence of amyloid in the immune system and in cells not forming part of the immune system as well.

This investigation was supported by a grant from *Rigsforeningen til Gigtens Bekæmpelse*

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* Each pharmacon in addition was administered to 5 mice treated with saline instead of casein No amyloid occurred in these animals

Acta path. microbiol scand Sect A, 82, 459–474, 1974

EXPERIMENTAL MURINE LEPROSY

2 Further Evidence for Varying Susceptibility of Outbred Mice and Evaluation of the Response of 5 Inbred Mouse Strains to Infection with *Mycobacterium lepraemurium*

Otto Closs and Olav A Haugen

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Female mice of the outbred strain NMRI, and the five inbred strains AKR, BALB/c, C3H, C57/BL, and White Label were infected with *Mycobacterium lepraemurium* Infectious lesions in the liver were evaluated histologically with regard to the type of inflammatory reaction and content of bacilli In the outbred strain the host response of individual mice varied considerably This variation diminished if a large inoculum was used and the infection allowed to proceed for a long time Within the inbred strains individual animals showed a more uniform type of reaction However, clear differences in the host response to murine leprosy could be demonstrated in these strains, most markedly between the strains C3H and C57/BL In C3H, the infection seemed to make uninhibited progress and the granulomas contained large numbers of bacilli with few or no surrounding lymphocytes The granulomas of C57/BL mice contained fewer bacilli and regularly showed infiltration by lymphocytes However after a large inoculum of bacilli this difference in host response seemed less evident The present study confirms our previous observation that outbred mice differ in their host response to murine leprosy Evidence is presented that this variation is due to differences in the ability of individual mice to mount a cell mediated immune response against the mycobacterium The capability to respond is shown to depend on genetic factors of the host

The clinical spectrum of human leprosy (*Ridley & Jopling* 1966) seems to depend on individual differences in cell mediated immune response (CMI response) against *Mycobacterium leprae* (*Turk & Bryceson* 1971) The basis for this variation in host reactivity is not known Therefore, it is of interest to establish an experimental model in which factors influencing the host response against mycobacterial infection may be studied in unmanipulated animals

We have reported on the clinical and histological evidence for varying susceptibility of outbred mice to infection with *Mycobacterium lepraemurium* (MLM) (*Closs & Haugen* 1973) The basis for this variation was assumed to be that outbred mice differ in their ability to mount an efficient cell mediated immune response against MLM This paper explores further the possibility of using murine leprosy as a model for the study of variations in host reactivity in mycobacterial infections

Received 7 ii 74   Accepted 7 ii 74

Requests for reprints should be addressed to Institute for Experimental Medical Research University of Oslo Ullevål Hospital Oslo 1 Norway

MATERIAL AND METHODS

Experimental animals Female mice of the outbred strain NMRI and the inbred strains C3H/A, C57/

TABLE 1 *Experimental Inoculation of* Mycobacterium lepraemurium *into Various Mouse Strains*

Series	Numbers	Strain	Dose of bacilli	Route of inoculation	Duration of infection (weeks)
1	1–8	NMRI	$2–8 \times 10^{9}$*	iv	5
2	1–8	„	„	„	11
3	1–9	„	6×10^{3}	„	12
8†	*1–4*	C3H	*$4–16 \times 10^{8}$**	ip	15
„	5–8	C57/BL	„	„	,
9	1–4	C3H	$4–16 \times 10^{7}$§	„	16
„	5–8	C57/BL	„	„	„

* Not actually counted but based on the assumption that the undiluted preparation of bacilli contained $1–4 \times 10^{10}$ bacilli/ml In subsequent countings of similar preparations the number of bacilli have been within this range

§ Dilution 1 10 of the suspension given to series 8

† *The other inbred strains, BALB/c,* AKR and White Label, were inoculated i p simultaneously with series 8 and 9, using the same doses of bacilli and killed after 19 weeks

BL/6J, BALB/c/A and AKR/A were used They were all obtained as specific pathogen free (spf) animals from Gl Bomholtgård Ltd, Denmark One additional inbred strain, designated White Label (WL) and non spf, was obtained from the Department of Pathology, Ullevål Hospital The animals were kept in cages, 4 8 mice in each, and fed on pellets (Norwegian Standard Stock No 1, Mice and Rats) and tap water *ad libitum*

Propagation of bacilli Mice of the NMRI strain were infected intravenously (iv) or intra peritoneally (ip) with 0 2 ml of a suspension of *Mycobacterium lepraemurium* Douglas strain (MLM) Sixteen to 20 weeks later, the animals were killed and their livers removed under sterile conditions

Harvesting of bacilli The liver was homogenized in a Tenbroek glass homogenizer after the addition of 10 ml 0 15 M NaCl (PS) The homogenate was centrifuged for 10 minutes at 100 × g, and the supernatant transferred to another tube and exposed to 10000 × g for 20 minutes The top layer of the pellet which was heavily contaminated *with liver cell organelles, was carefully washed off* The rest of the pellet was re suspended in 10 ml PS carefully avoiding any dark material at the bottom of the tube In this manner, a suspension of bacilli was obtained which was virtually free *from non acid fast material Clumps of bacilli* were removed either by sedimentation overnight at 1 × g in the cold room or by treating the suspension in a Branson sonifier Model B 12, (Branson Sonic Power Company Danbury, Connecticut, USA) at low output (50–80 W) for 10–15 sec

Counting of bacilli Bacilli were counted as described by *Hart & Rees* (1960) A small volume (0 004 ml) of the suspension was spread over an *8 mm diameter circle on a glass slide, using a* calibrated platinum loop After fixation and staining by Ziehl Neelsen's method, the bacilli were counted in 8 microscope fields per smear, usually 4 smears were prepared from each suspension

The number of bacilli per gram granuloma was calculated from the following equation

Bacilli per g granuloma =

$$\text{Bacilli per g liver} \times \frac{\text{AAG} \times \text{NG 50}}{\text{TA 50}}$$

where AAG = average area of one granuloma, NG 50 = number of granulomas per 50 microscope fields, and TA 50 = the total area of *50 microscope fields*

Experimental inoculation of mice Outbred mice were infected when 6–8 weeks old by intravenous (iv) inoculation into the tail vein, each animal receiving 0 2 ml of an MLM suspension from which clumps of bacilli had been removed Since it was necessary that the procedure should be consistent and that no bacilli should be deposited extra *vascularly, inbred mice were injected intraperi*toneally (ip) with 0 2 ml of the bacillary suspension An outline of the experimental groups are given in Table 1

Histological methods Tissue specimens were *fixed for approximately 12 hours in formol* alcohol containing 5 per cent glacial acetic acid, and then transferred to 80 per cent ethanol Further processing and embedding in paraffin was performed according to standard procedures Sections were stained with haematoxylin and eosin *(HE) and for* the demonstration of acid fast bacilli by the *Ziehl* Neelsen (ZN) technique

Histological evaluation All microscope slides

coded identification numbers only The HE and ZN stained sections were examined independently, and the code was broken when all sections had been examined The spread of infection to various organs was ascertained using both the HE and ZN stained sections Cellular reaction in the liver sections was recorded as previously described (*Closs & Haugen* 1973) Three main types of reactions were encountered 1) Granulomas consisting mainly of pale staining, granular macrophages without infiltration by lymphocytes or polymorphonuclear leucocytes, or in which the number of such cells was less than 5 This type of granuloma is shown in Fig 10 A 2) Granulomas surrounded by a rim of at least 5 lymphocytes (Fig 10 B) and 3) Granulomas showing an inflammatory exudate consisting of different cell types but with a predominance of polymorphonuclear leucocytes In each liver section, a total of 50 granulomas, selected at random, were recorded acording to this classification

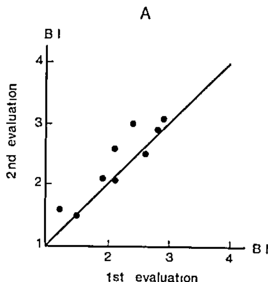
The Ziehl Neelsen stained liver sections were examined under oil immersion at a total magnification of 1250 × and the number of granulomas per microscope field, their diameter and bacillary content, were recorded The preparations were screened in the following way either 20 or 50 microscope fields, located 1 or 0 5 mm apart, were arbitrarily selected by using the millimeter scale on the crossboard The diameter of the granulomas was measured in μ by means of an ocular micrometer, and their mean diameter calculated on the basis of recordings of at least 30 granulomas According to an arbitrary scale, the amount of bacilli in each granuloma was recorded as either 1 — few, 2 = numerous (as shown in Fig 10 D), 3 abundant or 4 = packed (see Fig 10 C) The mean bacillary density was expressed by the bacillary index (BI) which represented the average of at least 30 recordings

Weighing procedures The animals were weighed immediately before they were killed Organ wet weight was determined on a Mettler H 5 analytic balance immediately after each organ had been removed from the animal and dissected free from fat and connective tissue The relative weights of the liver, spleen, and thymus were computed per 100 g body weight.

Statistical methods Wilcoxon s signed rank test (*Snedecor & Cochran* 1967) was used to test differences between paired measurements The correlation between the number of granulomas showing lymphocytic infiltration and the mean bacillary density was analysed with a Wang 700 A/B Electronic Computer (Wang Laboratories, Inc Tewksbury Mass, U S.A) equipped with a program for linear regression

Values of p below 0 05 were regarded as statistically significant

Reproducibility To test the reproducibility of the methods used for estimation of bacterial indices and the proportion of granulomas showing lymphocytic infiltration, the liver sections from all animals of series 3 were re-examined, after several weeks, by a blind technique The results have been plotted in Figs 1 A and 1 B The *method errors,*



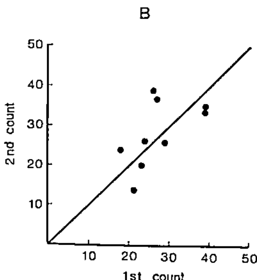


Fig 1 A Diagram showing the reproducibility of determination of the bacillary index (BI) in Ziehl Neelsen stained liver sections from a group of 9 outbred mice

B Diagram showing the reproducibility of the evaluation of cellular reaction in liver granulomas from 9 outbred mice Each point represents the proportion of 50 granulomas showing lymphocytic infiltration

i e the standard deviations of these parameters were computed from the usual formula $\sqrt{\frac{\Sigma D}{2n}}$ where D denotes the difference between duplicates and n the number of pairs compared The method error for the bacterial index was 0 21 and that for estimation of lymphocytic reaction 4 81 The coefficient of variation, therefore, can be calculated to be approximately 9 per cent and 17 per cent, respectively These results we regard to be within acceptable limits

RESULTS

Infection in outbred mice after intravenous inoculation Outbred mice killed 5 weeks after iv inoculation of 5×10^9 bacilli (series 1) showed lesions in the liver and spleen only The number of granulomas varied considerably, the greatest variation occurring in the spleen (Table 2) Clinically, all animals appeared unaffected by the infection at this stage

When infection of the liver was studied in greater detail, considerable individual variation was observed The number of granulomas in 20 high power fields and the proportion of granulomas containing numerous or abundant bacilli were higher in animals numbered 1-1, 1 2 and 1-7 than in the others (Fig 2) The same three animals deviated with regard to the type and degree of cellular reaction in the lesions As shown in Fig 3 the proportion of granulomas showing infiltration by small mononuclear cells was lowest in these animals

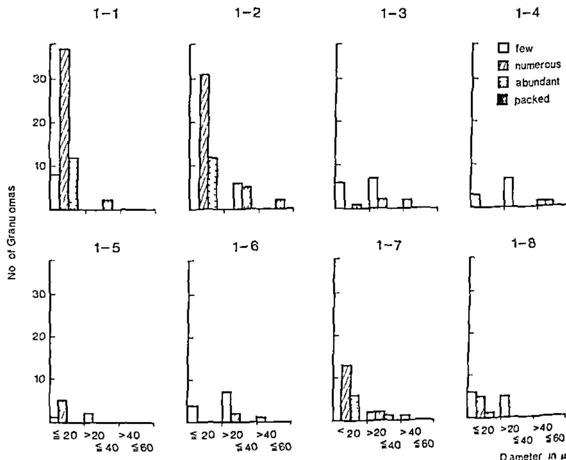


Fig 2 Histograms showing the number of granulomas per 20 microscope fields their diameter and bacillary content (shading of columns) Ziehl Neelsen stained liver sections from 8 outbred mice (series 1) infected 5 weeks previously with approximately 5×10^9 *M lepraemurium* iv

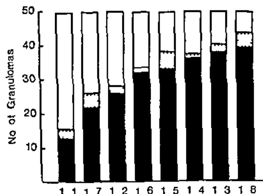


Fig 3 Cellular reaction in 50 liver granulomas from each of 8 outbred mice (series 1) injected 5 weeks previously with approximately 5 × 10[5] *M lepraemurium* Black, dotted and clear parts of the columns depict the number of granulomas with mononuclear cell reaction polymorphonuclear reaction or no reaction respectively

Eight mice killed 11 weeks after iv inoculation (series 2) showed further dissemination of the infection, lesions now appearing in a number of organs (Table 2) Their clinical condition still appeared to be unaffected In the liver, the number of granulomas per unit area showed less variation than in series 1 (15 24 versus 8 60) A considerable increase in the size of the granulomas had occurred

TABLE 2 *Occurrence of Granulomas at Various Sites in Outbred Mice 5 and 11 Weeks after Inoculation with 5 × 10[5]* M lepraemurium *intravenously*

Organ	5 weeks after inoculation (series 1)	11 weeks after inoculation (series 2)
Liver	8/8	8/8
Spleen	8/8	8/8
Lungs	0/8	3/7
Thigh muscle	0/8	3/8
Adrenals	0/8	2/6
Abdominal skin	0/8	2/8
Heart	0 8	1/8
Thymus	0/8	1/8
Kidneys	0 8	0 8

Numerator indicates number of animals with positive findings, denominator indicates number of animals from which histological sections were examined

during the 6 week period (Table 3) The amount of bacilli in the lesions as expressed by the BI (bacillary index) had also greatly increased Some variation in these parameters in the individual animals could be demonstrated (Fig 4) In animals numbered 2 2 and 2 5, the majority of the lesions were smaller than 60 μ and tended to contain large amounts of bacilli In contrast, in animals numbered 2 3 and 2-7 more than half of the lesions were larger than 60 μ and they also contained comparatively less bacilli The proportion of granulomas showing no inflammatory reaction was increased in comparison with those in mice killed after 5 weeks, more than half of the granulomas now lacked infiltration by small mononuclear cells (Fig 5) Some individual variation in the degree and type of cellular reaction occurred but this was less prominent than in the former group of mice However, in the livers of animals numbered 2 2 and 2 5, which contained small lesions and many bacilli, the lack of cellular reaction was most prominent

In animals injected iv with a smaller dose of bacilli (6 × 10[c]) and killed after 12 weeks (series 3), the degree of infection was assessed and the number of bacilli per liver counted, considerable individual variation was again observed Some variation was also noted in the relative weights of the liver, spleen and thymus (Table 4) Enlargement of the liver and spleen seemed to be roughly proportional to the stage of the infection All animals showed involvement of the liver, all but one showed granulomas or bacilli in the spleen, whereas only one animal showed signs of infection elsewhere In the liver the size distribution of the granulomas was similar to that seen in series 1 (Table 3)

The number of bacilli per liver showed considerable individual variation (Table 4) Animals numbered 3-4 3 5 and 3 2 had substantially larger amounts of bacilli in the liver than animals numbered 3 6 and 3 9 Pilot experiments had shown that in this strain approximately 80 per cent of a similar dose of bacilli injected iv may be recovered from the liver after 24 hours Assuming that 80 per

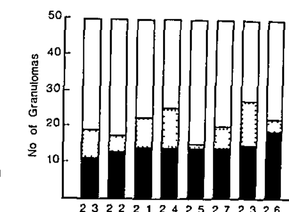


Fig 5 Cellular reaction in 50 liver granulomas from each of 8 outbred mice (series 2) injected *11 weeks previously with approximately 5 × 10⁹ M lepraemurium* The black, dotted and clear parts of the columns depict the number of granulomas with mononuclear cell reaction polymorphonuclear reaction or no reaction respectively

cent of the injected bacilli were taken up by the liver initially in all mice, an average doubling time for their multiplication in this organ could be calculated As shown in Table 4, *the average doubling time varied between* 9 2 and 12 8 days

Histological evaluation of the extent of infectious involvement of the liver and the degree and type of cellular reaction again revealed a spectrum The type of cellular reaction encountered in the liver granulomas of series 3 is shown in Fig 6 In two animals (3 6 and 3 1), nearly 80 per cent of the lesions showed infiltration by lymphocytes *The lymphocytic reaction was less pronounced* in the other animals and in two of *them (3 7 and 3 2) more than half of* the lesions completely lacked this type of reaction

Some variation in the number of granulomas per unit area of liver tissue was seen (Table 5) When 30 liver granulomas from each of the 9 animals were examined their

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Fig 4 Histograms showing the size distribution of 30 liver granulomas and their content of bacilli in 8 outbred mice (series 2) killed 11 weeks after inoculation with approximately 5 × 10⁹ *M lepraemurium* The amount of bacilli in the granulomas is indicated by the shading of the columns

TABLE 3 *Size Distribution of Liver Granulomas Recorded in Outbred Mice in Relation to Inoculum Size and Time after Infection with* M lepraemurium

Diameter of granulomas (μ)		5 × 10⁹ bacilli		6 × 10 bacilli
		5 weeks (series 1)	11 weeks (series 2)	12 weeks (series 3)
	≦ 25	199	65	221
> 25	≦ 50	179	184	172
> 50	≦ 75	22	103	15
> 75	≦ 100	0	29	0
> 100	≦ 125	0	3	0
Numbers recorded		400	384	408

TABLE 4 *Relative Organ Weights, Number of Bacilli per Liver and their Doubling Time in Outbred Mice infected with* M lepraemurium*

Animal No	Relative organ weights§			No of bacilli per liver × 10^7	Average doubling time, days
	Liver	Spleen	Thymus		
3-1	8 188	2 214	162 3	160	10 4
3-2	8 312	2 018	119 5	210	9 4
3-3	6 890	1 179	259 6	97	11 4
3-4	7 336	1 765	203 2	330	9 2
3-5	8 072	1 819	196 2	195	10 1
3-6	6 611	1 100	175 1	55	12 8
3 7	7 277	1 450	217 9	160	10 4
3-8	7 784	1 538	207 6	140	10 6
3 9	7 190	1 975	187 7	64	12 3
Mean infected animals	7 518	1 673	192 1		
Mean 4 controls	5 053	0 434	234 4		

* Inoculated with 6 × 10^9 bacilli iv 12 weeks previously
§ Liver and spleen weights as g/100 g body weight, thymus weight as mg/100 g body weight

size and bacillary content was found to vary as shown in Fig 7 In some animals (3-1, 3 6), the lesions were relatively large and contained few bacilli while in others (3-7, 3 8), mainly small lesions with large amounts of bacilli were found However, in animals 3-4 in which the largest number of bacilli in the liver was found, the granulomas were relatively large and contained many bacilli As a measure of the amount of bacilli per lesion, the BI correlated extremely well with the calculated number of bacilli per gram granuloma (r = 0 97) The animals which had few bacilli in their lesions as expressed by a low BI (Table 5) also had the highest number of granulomas with a mononuclear cell reaction The inverse relationship between these two parameters in the mice of series 2 and 3 is illustrated in Fig 8

Infection in inbred strains after intraperitoneal inoculation Five different inbred strains of mice, C3H, C57/BL, BALB/c, WL and AKR were inoculated ip with one of two different doses of MLM The intraperitoneal route was chosen since clean and consistent iv injection was difficult to ensure in some of the strains because of small tail veins The animals were killed after a period varying from 16–19 weeks

Among the animals receiving the largest

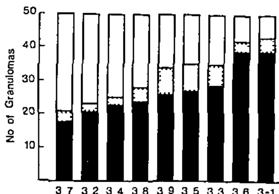


Fig 6 Cellular reaction in 50 liver granulomas from each of 9 outbred mice (series 3) killed 12 weeks after iv inoculation with 6 $\times$ 10^{8} *M le praemurium* Black, dotted, and clear parts of the columns indicate the number of granulomas with mononuclear cell reaction, polymorphonuclear re action, or no reaction, respectively

dose of bacilli (series 8), some strain differences could be observed clinically Judged by signs of wasting paralysis, degree of spontaneous activity and fur quality, C57/BL appeared to be the strain least affected by the infection The most severe clinical signs of illness were observed in the C3H strain These animals all showed considerable wasting, paresis of the hind limbs was frequent and complete paralysis occurred in one animal

Liver sections from all infected animals were screened for the presence of granulomas and the degree and type of cellular reaction encountered in them The results are given in Table 6 From these data and the clinical observations it appeared that the largest difference in the course of the infection was that between the strains C3H and C57/BL We therefore decided to concentrate our further studies on these strains

C3H and C57/BL mice inoculated intra

Fig 7 Histograms showing the size distribution of 30 liver granulomas and their bacillary content examined in each of 9 outbred mice (series 3), killed 12 weeks after iv inoculation with 6 $\times$ 10^{8} *M lepraemurium* The amount of bacilli present in the granulomas is indicated by the shading of the columns

TABLE 5 *Liver Involvement in* M lepraemurium *infected Outbred Mice Comparison of Histological Criteria and Bacillary Counts*

Animal No	No of bacilli per g liver × 10^7	No of granulomas per 50 M F	Average diameter of granulomas in μ	B G	B I
3 1	55	13	52 2	976	1 5
3 2	59	11	41 7	1,942	2 1
3–3	42	16	31 2	1,548	2 1
3–4	150	15	43 4	3,167	2 6
3 5	77	15	39 3	2,003	1 9
3–6	25	8	68 7	402	1 2
3–7	67	10	38 5	2,645	2 4
3 8	46	10	29 2	3,377	2 9
3 9	28	12	30 8	1,612	1 8

M F = Microscope fields B I = Bacillary index B G – Number of bacilli per g granuloma × 10^7

TABLE 6 *The inflammatory Reaction in Liver Granulomas in 5 Inbred Strains of Mice infected with* M lepraemurium*

Strain	No of animals	Inflammatory reaction			Total No of granulomas evaluated
		M	P	0	
C3H	4	15	20	165	200
BALB/c	4	41	65	94	200
AKR	4	63	33	104	200
WL	4	74	46	80	200
C57/BL	4	166	28	6	200

M = predominance of lymphocytes P = predominance of polymorphonuclear leucocytes 0 = no inflammatory cells present

* Inoculated with 10^8 bacilli i p 16 weeks (C3H, C57/BL) or 19 weeks (BALB/c, AKR, WL) previously

peritoneally with 10^8 bacilli (series 9) were examined histologically 16 weeks later for dissemination of the infection The spread of infection to various organs was similar in the two strains, except for the spread to the thymus where some bacilli were found in every C57/BL while this organ appeared to be free from infection in C3H mice (Table 7) So far, the significance of this observation remains uncertain In the non lymphoid organs, where inflammatory reactions were readily detectable, mononuclear cell infiltration was seen in the C57/BL strain only The relative liver weights showed the largest increase in C57/BL while the most pronounced enlargement of the spleen was observed in C3H (Table 8)

The lesions in the liver were studied in greater detail In C3H mice, the number of lesions per high power field was significantly larger than that in C57/BL mice (Fig 9) Also, in C3H, the majority of the lesions had a diameter smaller than 40 μ, whereas most of them were larger than 40 μ in the C57/BL strain The lesions in C57/BL usually consisted of a number of macrophages containing relatively few bacilli, while single, enlarged macrophages packed with bacilli were frequently seen in C3H, the larger aggregates being uncommon In addition to the lesions

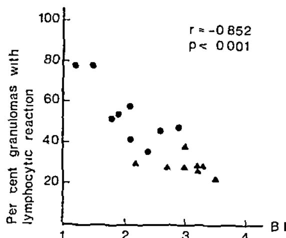


Fig 8 Scatter diagram showing the correlation between cellular reaction and bacillary density (BI) in liver granulomas of *M lepraemurium* infected outbred mice Animals of series 2 (▲) were killed 11 weeks after inoculation with approximately 5 × 10^{8} bacilli, and series 3 (●) were killed 12 weeks after receiving 6 × 10^{9} bacilli ıv

TABLE 7 *Occurrence of Granulomas at Various Sites in C57/BL and C3H Mice 16 Weeks after ip Injection of* 10^{9} M lepraemurium

Organ	C57/BL	C3H
Liver	4/4	4/4
Spleen	4/4	4/4
Lungs	2/4	0/4
Kidneys	0/4	0/4
Thymus	4/4	0/4
Heart	0/4	1/4
Thigh muscle	0/4	0/4
Adrenal	1/2	2/3
Abdominal skin	3/4	4/4
Abdominal lymph node	4/4	3/4
Peripheral lymph node	2/2	4/4

Numerator indicates number of animals in which findings were positive, denominator indicates number of animals from which histological sections were examined

accounted for in Fig 9, a number of scattered small macrophages containing only a few bacilli were seen in the livers of C3H mice It was likely that these cells had recently ingested their bacilli, indicating that infection was still spreading in this strain Calculation of the mean diameter of liver granulomas and evaluation of their bacillary content expressed as the BI (Table 8) confirmed the difference between the two strains The mean

TABLE 8 *Diameter of Liver Granulomas and their Content of Bacilli in Mice infected with* Mycobacterium lepraemurium*

Animal No	Strain	Relative organ weights§			Diameter of granulomas in μ	B I
		liver	spleen	thymus		
9 1	C3H	6 698	3 267	120 7	26 3	3 4
9–2	,	5 929	1 619	141 1	20 2	3 5
9 3		6 634	3 109	117 6	30 5	3 5
9 4	»	5 917	1 564	128 5	21 2	3 4
Mean		6 295	2 390	127 0	24 6	3 5
9–5	C57/BL	9 223	2 469	190 7	58 6	2 6
9 6		7 210	1 896	183 8	86 3	1 4
9 7	,	8 870	2 145	187 8	77 0	2 4
9 8	,	6 789	2 201	114 7	86 2	2 2
Mean		8 023	2 178	169 3	77 0	2 2
Controls	C3H	5 586	0 257	136 8		
(Mean of 4 mice)	C57/BL	5 542	0 594	221 3		

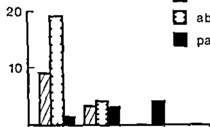
B I = bacillary index

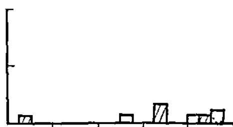
* Inoculated with 10 MLM 16 weeks previously

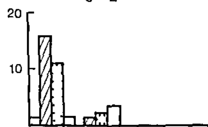
§ Liver and spleen weights as g/100 g body weight, thymus weight as mg/100 g body weight

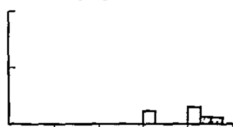
C3H

C57 BL

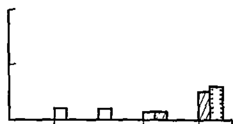


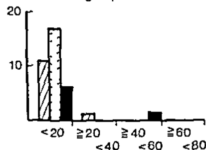












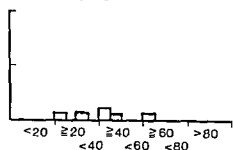
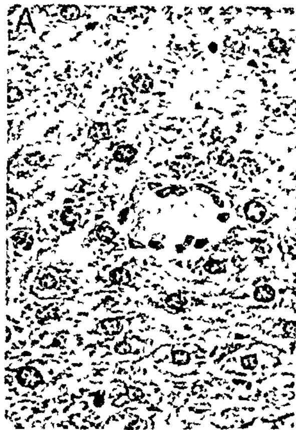
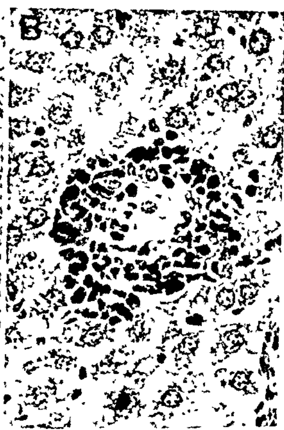
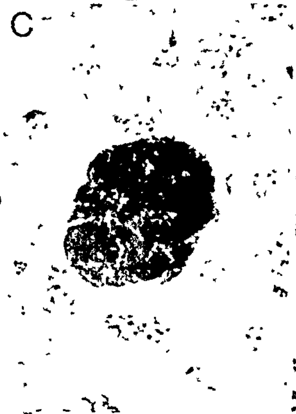
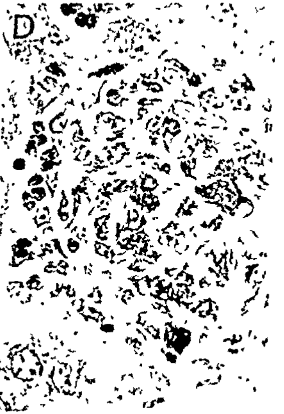


Fig 9 Histograms showing the number of liver granulomas per 20 microscope fields their size distribution and content of bacilli (shading of columns) in the two inbred mouse strains C3H and C57/BL The animals were killed 16 weeks after receiving 10^{8} *M lepraemurium* ip


A


B


C


D

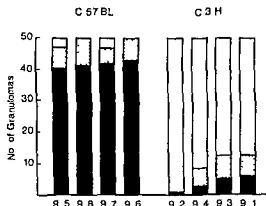


Fig 11 Cellular reaction in the liver granulomas of the inbred mouse strains C3H and C57/BL Four animals of each strain and 50 examined granulomas from each animal Black, dotted, and clear parts of the columns indicate the number of granulomas with mononuclear cell reaction, poly morphonuclear reaction, or no cellular reaction, respectively

granuloma diameter in C3H was 24 6 μ and 77 0 μ in C57/BL mice The mean values for the BI were 3 5 and 2 2, respectively

The typical lesions in C3H mice did not contain small mononuclear cells (Fig 10 A) while an abundance of such cells could be found in C57/BL lesions (Fig 10 B) A comparison of the two strains regarding the type of cellular reaction in the liver granulomas is given in Fig 11 Among the examined 200 granulomas from C57/BL mice, less than 5 per cent were devoid of inflammatory reaction More than 80 per cent of the granulomas in C57/BL showed infiltration by lymphocytes In C3H mice on the other hand, more than 75 per cent of the granulomas lacked any kind of inflammatory reaction, and less than 10 per cent showed infiltration by lymphocytes In contrast to the pronounced difference between the strains, a relatively uniform picture was observed within each strain In mice receiving 10^9 bacilli (series 8), the infection had spread to a number of organs in both strains The enlarged livers of these severely infected animals contained numerous granulomas which also had a somewhat different histological appearance in the two strains The granulomas encountered in C57/BL mice were, on the whole, larger and tended to coalesce, forming elongated infiltrates along the hepatic vessels Areas of central necrosis were frequent, leaving only shadows of macrophages and cellular debris The granulomas of C3H mice were scattered more diffusely throughout the liver parenchyma and the architecture of the granulomas appeared to be less organized The size of the individual macrophages also appeared to be slightly larger in this strain in comparison with the C57/BL An abundance of bacilli were present in the lesions of both strains Typically, the central part of the granulomas in C57/BL mice, contained few bacilli, while there was an even density of bacilli throughout the granulomas in C3H mice The number of bacilli per cell also appeared to be much higher in the lesions of C3H mice Infiltration by polymorphonuclear leucocytes were found in the lesions of both strains, but were more prominent in C3H mice Some plasma cells were also found in the granulomas of both strains Signs of infiltration by small mononuclear cells were virtually absent in C3H and not very marked in C57/BL animals Thus, at this stage of widespread infection, the differences between the two strains were altogether less clear-cut, and the bacilli seemed to multiply vividly in both strains

Fig 10 A Typical lesion from the liver of a C3H mouse, showing an aggregate of macrophages without any inflammatory reaction B Liver lesion from a C57/BL mouse with central macrophages and a marked inflammatory reaction, mainly consisting of small lymphocytes HE × 265 C Granuloma packed with bacilli bacillary index 4 D granuloma with numerous bacilli bacillary index 2 ZN × 425

DISCUSSION

Even if an inflammatory reaction is comprised mainly of mononuclear cells it is not justifyable to conclude that it represents an immune reaction of the delayed type (CMI reaction) (*Vasalli & McCluskey* 1971) How-

ever, the triggering of a CMI reaction requires the presence of specifically activated T-lymphocytes. Therefore the small lymphocyte may be regarded as a *sine qua non* histological marker for this type of immune reaction. In human leprosy, clear-cut histological differences between the polar forms of the disease are in evidence (*Ridley & Jopling* 1966). Bacilli are rarely encountered in tuberculoid lesions, while numerous small lymphocytes and epitheloid cells are present. Lepromatous lesions, on the other hand, contain large amounts of bacilli, but there is a conspicuous absence of lymphocytes. These histological differences have been shown to correlate with the results of *in vivo* and *in vitro* tests for CMI against *M. leprae* (*Myrvang et al.* 1973). Together, the histological and immunological data indicate that the clinical course of leprosy depends on the ability to mount and maintain a CMI-response against the mycobacterium.

Since, like *M. leprae,* the causative microorganism in murine leprosy is an obligate intracellular parasite, CMI may be expected to play an important role in the host defence against this infection (*Mackaness* 1971). In the present study of murine leprosy, the degree of host response has been evaluated by histological criteria. The proportion of liver granulomas showing lymphocytic infiltration was used as an indirect measure of the CMI response in individual mice. The amount of bacilli in the lesions were expressed by the bacillary index, based on an arbitrary scale. It emerged that in outbred mice there is an inverse and statistically significant relationship between the mean bacillary index and the proportion of granulomas showing infiltration by lymphocytes. The same inverse relationship is also valid in inbred strains. This strongly suggests that CMI is a major protective mechanism in mice infected with *M. lepraemurium*.

The host reaction to MLM-infection, measured by histological criteria, was found to vary in outbred mice. The possibility that this variation might be due to inaccuracies implied in the histological evaluation was carefully considered. The method error was however, shown to be within acceptable limits. Since variation observed in the outbred strain was significantly different from that observed in inbred strains it cannot be explained on the basis of method errors alone. The fact that only a relatively small area of each liver was examined increases the possibility of sampling errors. However, a high degree of correlation between the mean bacillary index and the calculated number of bacilli per gram granuloma tissue was found. As the former figure depended on the examination of one single liver section while the total number of bacilli per liver was included in the calculation of the latter, the strong correlation between them indicates that the sections may be regarded as true random samples. Based on these considerations we conclude that the observed variation reflects true differences in the ability of individual mice to mount a host reaction against the invading mycobacterium.

The severity of experimental murine leprosy seems mainly to depend on three factors. The size of the inoculum, the time elapsed after inoculation, and the resistance of the host. The expression of host resistance, however, is clearly influenced by the other two factors, individual differences in host resistance were for instance more difficult to demonstrate in animals given a large dose of bacilli than in those receiving a smaller inoculum.

In mycobacterial infections, an effective host defence seems to depend on the presence of specifically sensitized lymphocytes that are able to activate the macrophages locally in the lesions (*Dannenberg et al.* 1968, *Mackaness* 1968). Regarding MLM infection, it may be postulated that the larger the number of granulomas the smaller the number of specifically competent lymphocytes initially available per lesion. In animals with a large number of granulomas in the liver we observed that a smaller proportion of the lesions showed lymphocytic infiltration in comparison with animals in which the liver contained less granulomas (series 1, Figs. 2 & 3).

When the number of MLM in the ino-

culum is increased, the infection seems soon to reach a level where host resistance becomes ineffective. The CMI reaction observed in outbred mice infected with a relatively large dose of bacilli became more uniform in all the animals as the infection progressed, the liver lesions showing gradually decreasing lymphocytic infiltration. The relatively high bacillary density found in the outer zone, as opposed to the central parts of the granulomas in the C57/BL mice of series 9, might similarly reflect that an alteration in the microbicidal capacity of the macrophages has occurred at some stage during infection. This may indicate that host defence mechanisms become suppressed as a result of the infection. As shown by *Ptak et al* (1970), MLM-infection induces a general depression of the CMI-response. The use of smaller inocula, i.e. 10^6 bacilli or less, may reduce this depressive effect and allow a more precise evaluation of the capability of individual mice to react against this mycobacterium.

In attempts to fit host resistance in murine leprosy into the clinical and immunological spectrum of human leprosy it seems as if the whole range of reactions in mice is displayed towards the "lepromatous end". In his original work on murine leprosy, *Stefansky* (1903) described two forms of the disease in wild rats, the mild localized glandular form, and the severe disseminated musculocutaneous form. However, the two forms of the disease were never established under experimental conditions. Although it has been reported that, following experimental inoculation into outbred mice and rats (*Fite* 1940), the course of the infection may vary in different animals, all these animals sooner or later died from the infection. It is relevant to stress that none of the mice we have studied so far had a host resistance strong enough to stop the proliferation of MLM. Therefore under the experimental conditions used, murine leprosy seems invariably to have a fatal outcome. It was not until histological criteria were used that the spectrum of host response in outbred mice became apparent.

The study of inbred strains revealed that, in contrast to the varied picture exhibited by outbred mice, all animals of an inbred strain showed essentially the same type of reaction. However, considerable strain differences were observed. This strongly suggests that the ability of mice to react to MLM infection depends on genetic factors.

Among the 5 inbred strains studied, the strongest and the weakest reactions were seen in strains C57/BL and C3H, respectively. The polar types of reaction seen in these two strains agrees with the findings by *Kawaguchi* (1959) who classified murine leprosy into a benign and a malignant form on the basis of the appearance of subcutaneous lepromas in the C57/BL and C3H strains of mice. Furthermore, the responses seen in these two strains seem to correspond to the extremes of the spectrum present in the outbred strain NMRI, even among the limited number of outbred mice studied, animals that responded nearly as well as C57/BL or as poorly as C3H mice were found. While the lack of reaction seen in C3H mice is reminiscent of lepromatous leprosy in man, the histological picture of the lesions in the C57/BL strain has several features in common with that seen in human borderline leprosy (within the spectrum BB to BT). In all C57/BL mice receiving 10^8 bacilli ip, the lymphocytic infiltration in the liver granulomas was considerable. In comparison with the lesions in C3H mice, less bacilli were present and in some granulomas none at all were visible. If the inoculum contained 10 times as many bacilli, the bacillary content of the lesions increased considerably and the lymphocytic reaction became less pronounced. Hence it is conceivable that if instead of increasing the inoculum it were reduced to 10, 10^6 bacilli, or even less, there would be more specifically competent lymphocytes available per lesion and the resistant strain would become more able to destroy the MLM. This would further extend the spectrum of this model towards the "tuberculoid type" of reaction.

On the basis of the present study we con-

clude that a spectrum of host resistance, depending on a genetically determined ability to mount a CMI-response against MLM, is present in outbred mice The polar ends of this spectrum are represented by the inbred strains C3H and C57/BL On this basis murine leprosy may become an important experimental model for the study of host resistance in mycobacterial infection

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This work was supported by grants from *Anders Jahre's Fund* for the Promotion of Science and *The Norwegian Research Council for Science and the Humanities*

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AUTORADIOGRAPHIC STUDIES OF EXPERIMENTALLY INDUCED ATYPIAS IN THE CERVICAL EPITHELIUM OF MICE

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The autoradiographic pattern of the cervix epithelium after iterated application of the carcinogen 3,4 benzpyrene was investigated in 100 C57Bl mice There was a gradual increase in the proportion of labelled cells with increasing degree of atypias Labelling of superficial layers occurred in almost all cases with severe atypias (grade III) and in 8 per cent of those with moderate atypias (grade II) The labelling of superficial cells in moderate atypias (grade II) suggests that, despite cytoplasmic maturation, their nuclei retained the property of less mature or of undifferentiated cells, i e the capacity to synthesize DNA The highest number of labelled cells was found in mice presenting severe atypias with epithelial buds protruding into the stroma

Spontaneous carcinomas of the cervix are extremely rare in mice (*Murphy* 1966) However, the mouse has become the laboratory animal of choice in the induction of cervical tumours after local or systemic treatment with hormones and/or carcinogens (*Reagan et al* 1955, *Scarpelli* & *von Haam* 1957, *Gardener* 1959, *Koprowska* & *Bogacz* 1959, *Murphy* 1961, *Wentz* 1962, *Dunn* 1963, *Laffargue et al* 1963, *Iijima et al* 1964, *Kaminetzky* & *Swerdlow* 1964, *Yong* & *Campell* 1965, *Kaminetzky* 1966, *Kehar* & *Wahi* 1967, *Graham* 1970, *Forsberg* & *Breistein* 1972 and *Joneja* & *Coulson* 1973) Some of these reports are concerned with the development of epithelial atypias preceding invasive carcinoma They were reviewed by *Scarpelli* & *von Haam* (1960)

In previous publications (*Rubio* & *Lagerlof* 1972, 1973) we reported the histological characteristics of intraepithelial atypias of the uterine cervix in C57Bl mice after local application of a carcinogen, 3,4 benzpyrene After 5 months' application of the carcinogen, 94 per cent of the 93 mice with atypias had irregular epithelial buds protruding into the stroma, while the remaining 6 per cent had a smooth border to the underlying stroma Moreover, intra-epithelial atypias with buds were present in all the 16 mice which also had developed invasive carcinoma of the cervix

Similarly, 95 per cent of 33 patients in whom a development of cervical invasive carcinoma was found at follow-up had in preceding biopsies epithelial buds with carcinoma *in situ* (*Rubio* & *Soderberg* 1972) Moreover, 95 per cent of 220 cases with micro-invasive carcinoma had in addition associated lesions showing epithelial buds with carcinoma *in situ* (*Rubio* & *Söderberg* 1969)

The purpose of the present investigation was to provide information about the autoradiographic pattern of the atypical epithelium in mice, with special reference to the disclosure of possible differences between the

Received 6 xii 73 Accepted 8 ii 74

TABLE 1 *Incorporation of ³H Thymidine into the Various Compartments of the Cervical Epithelium in 93 Mice Treated with 3 4 Ben pyrene*

	Histology												
Time of *application*	Normal (*21 mice*)			Atypias grade I (*18 mice*)			Atypias grade II (*35 mice*)			Atypias grade III (*19 mice*)			Total
	BP	IM	S	BP	IM	S	BP	IM	S	BP	IM	S	
1 month	10	–	–										10
2 months	4	–	–	5	3	–							9
3 months	2	–	–	5	2	–	1	1	–				8
4 months				4	2	–	1	1	–				5
5 months	5	–	–	4	3	–	33	29	3	19	19	18	61
Total	21	–		18	10	–	35	31	3	19	19	18	93

BP denotes the basal parabasal compartment IM the intermediate compartment and S the superficial compartment

proportions of cells in the phase of DNA synthesis In pursuit of this end, various degrees of intra epithelial atypias, with and without epithelial buds have been investigated

MATERIAL AND METHODS

A) *Mice Treated with 3 4 Benzpyrene*

The cervix portio area in 93 C57Bl mice was painted twice a week with a one per cent solution of 3 4 benzpyrene in acetone The treatment was performed during one month in 10 mice during two months in 9 mice during three months in 8 mice during four months in 5 mice and during five months in the remaining 61 mice

B) *Spayed Mice Treated with benzpyrene*

Seven mice were ovariectomized bilaterally Two weeks after ovariectomy the cervix portio of the animals was painted twice a week with benzpyrene during 1 2 4 and 5 months Six animals in groups of 2 were sacrificed during months 1 2 and 4, the seventh during month 5

C) *Acetone Treated Controls*

A third group comprising 24 mice were painted with one per cent acetone twice a week during 1 to 5 months Four were sacrificed the 1st month and groups of 5 during the following months

Processing of the Cervical Tissue

After the alloted time of application of benzpyrene or acetone the animals were sacrificed The cervix portio area was removed and incubated during one hour at 37° C in 1 ml of Parker 199 medium containing 1 microCu ³H thymidine (specific activity 6 7 Cu/mMol), as proposed by Richart for the human uterine cervix (Richart 1963) The tissue was then washed twice in saline and



Fig 1 Normal epithelium Only cells in the basal parabasal compartment are labelled

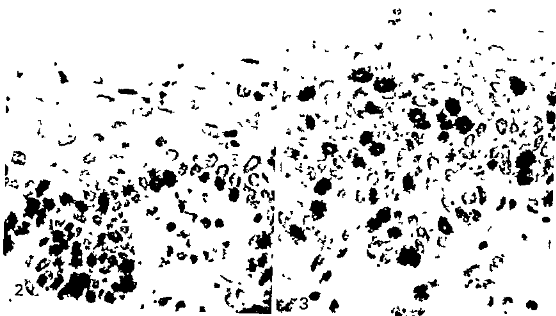
Fig 1 5 Haematoxylin stained autoradiographic preparations (300 ×)

Fig 2 Grade I atypia Cells in the basal parabasal and intermediate compartments in one bud are labelled

Fig 3 Grade II atypia The epithelial stroma border is uneven with bud formation *Cells in the* basal parabasal intermediate and superficial compartments are labelled

Fig 4 Grade III atypia without buds Cells in all three compartments are labelled

Fig 5 Grade III atypia with buds Heavy labelling of cells in all three compartments



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TABLE I *Incorporation of ³H Thymidine into the Various Compartments of the Cervical Epithelium in 93 Mice Treated with 3 4 Benzpyrene*

Time of application	Normal (21 mice)			Atypias grade I (18 mice)			Atypias grade II (35 mice)			Atypias grade III (19 mice)			Total
	BP	IM	S	BP	IM	S	BP	IM	S	BP	IM	S	
1 month	10	–	–										10
2 months	4	–	–	5	3	–							9
3 months	2		–	5	2	–	1	1	–				8
4 months				4	2	–	1	1					5
5 months	5	–	–	4	3	–	33	29	3	19	19	18	61
Total	21	–	–	18	10	–	35	31	3	19	19	18	93

Histology

BP denotes the basal parabasal compartment IM the intermediate compartment and S the superficial compartment

proportions of cells in the phase of DNA synthesis In pursuit of this end, various degrees of intra epithelial atypias, with and without epithelial buds have been investigated

MATERIAL AND METHODS

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The cervix portio area in 93 C57Bl mice was painted twice a week with a one per cent solution of 3 4 benzpyrene in acetone The treatment was performed during one month in 10 mice during two months in 9 mice during three months in 8 mice during four months in 5 mice and during five months in the remaining 61 mice

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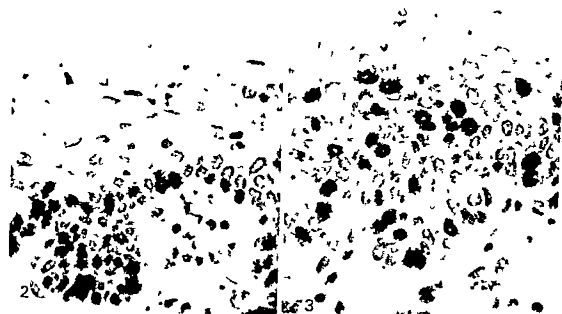
Fig 1–5 Haematoxylin stained autoradiographic preparations (300 ×)

Fig 2 Grade I atypia Cells in the basal parabasal and intermediate compartments in one bud are labelled

Fig 3 Grade II atypia. The epithelial stroma border is uneven with bud formation *Cells in the* basal parabasal intermediate and superficial compartments are labelled

Fig 4 Grade III atypia without buds *Cells in all* three compartments are labelled

Fig 5 Grade III atypia with buds Heavy labelling of cells in all three compartments



4 5

TABLE 2 *Incorporation of ³H Thymidine into the Various Compartments of the Cervical Epithelium in 7 Oophorectomized Mice Treated with 3,4 benzpyrene*

	Histology		
	BP	IM	S
Normal	3	–	–
Grade I atypia	4	2	–
Total	7	2	–

BP denotes the basal parabasal compartment, IM the intemediate compartment and S the superficial compartment

TABLE 3 *Incorporation of ³H Thymidine into the Various Compartments of the Cervical Epithelium in 24 Mice Treated with Acetone*

	Histology		
	BP	IM	S
Normal	14	–	–
Grade I atypia	10	5	–
Total	24	5	–

BP denotes the basal parabasal compartment, IM the intermediate compartment and S the superficial compartment

fixed in 10 per cent neutral formalin Thirty or more 3 micrometer thick sections were done along the oral-caudal axis, following which they were coated with Kodak stripping film AR 10 and exposed 18 to 22 weeks in light tight boxes at 4° C. The films were developed in Kodak D19 B solution for 5 minutes at 20° C, fixed in Kodak X ray Fix and finally stained in Harris Haematoxylin during 2 minutes Alternative sections were done for conventional histological studies They were stained with Haematoxylin and Eosin

Histological Considerations

After local application of 3,4-benzpyrene, the cervical epithelium developed various degrees of cellular alteration As in previous publications (*Rubio* & *Lagerlof* 1973), those alterations were classified according to increased degree of atypia in atypia grades I, II and III The thickness of the squamous epithelium of the cervix was arbitrarily divided into three equal parts BP) a basal parabasal zone, IM) an intermediate zone and S) a superficial zone bordering the lumen The occurrence of labelled cells in each part was recorded. If buds were present it might often be difficult to divide the epithelium into 3 equal parts. The following arbitrary division was therefore applied Labelled basal and parabasal cells or cells replacing those two layers were considered as belonging to the BP compartment, all other labelled cells within the bud being allotted to the IM compartment. Labelled cells in the superficial epithelium on the top of buds were classified as belonging within the S zone. In order to record the presence of

TABLE 4 *The Percentage of ³H Thymidine Labelled Cells in the 3 Zones of the Normal or Atypical Cervical Epithelium of Mice Treated with 3,4 Benzpyrene*

	Histological zones					
	Basal parabasal		Intermediate		Superficial	
Histology	No of labelled cells/ total no of cells	Per cent (range)	No of labelled cells/ total no of cells	Per cent (range)	No of labelled cells/ total no of cells	Per cent (range)
Normal (3 mice)	156/1103	14% (13%–17%)	0/457	0% (–)	0/282	0% (–)
Grade I (3 mice)	478/1873	26% (15%–39%)	106/1283	8% (7%–11%)	0/1059	0% (–)
Grade II (3 mice)	631 1639	38% (28%–48%)	156/1209	13% (6%–19%)	805/3783	21% (13% 28%)
Grade III (5 mice)	233/865	37% 31%–51%)	584/2106	28% (8%–21%)	1771/6412	28% (21%–30%)

TABLE 5 *The Percentage of ³H Thymidine Labelled Cells in Normal and Atypical Epithelium of the Cervix Mice Treated with 3 4 Benzpyrene*

Histology	Epithelium without buds		Epithelium with buds	
	No of labelled cells/total no of cells	Per cent (range)	No of labelled cells/total no of cells	Per cent (range)
Normal (4 mice)	250/2657	9% (7%–12%)	116/886	13% (13%–14%)
Grade I atypia (3 mice)	210/1485	14% (8%–17%)	257/987	26% (21%–28%)
Grade II atypia (3 mice)	159/756	21% (11% 24%)	939/2111	44% (39%–50%)
Grade III atypia (4 mice)	511/1950	26% (21%–32%)	1409/3134	45% (34%–51%)

For each mouse areas with or without buds have been included in the appropriate column

labelled cells in each compartment the thickness of the ep thelium in 28 mice was also divided into three equal parts by the aid of an ocular scale Areas showing the highest proportion of labelled cells were then selected for quantitative determina tions The percentage of labelled cells among five hundred or more consecutive cells was recorded as the labelling index

Determinations of the percentage of labelled cells were performed separately in areas with intra epi thelial atypias respectively with and without buds A cell having a nucleus covered by at least 5 grains was considered as labelled

Determination of the Oestrous Cycle

The changes produced in the cervical epithelium by the oestrous cycle were recorded in H and E stained histological sections in 86 of the 93 mice They were divided in pro oestrous oestrous, meta oestrous and di-oestrous following the criteria pre sented by *Bartelanfy* (1963) In all 86 mice, nor mal epithelium was observed in add tion to atypical epithelium In the remaining 7 mice the cycle could not be determined because of overgrowth of atypical epithelium or invasive carcinoma The results of the autoradiographical investigation were correlated to the histologically identifiable phase of the oestrous cycle

RESULTS

Qualitative Determinations

A) *Mice treated with 3,4 benzpyrene* (Figs 1 to 5) The results of qualitative determina tions of nuclear labelling are shown in Table 1 It is seen here that nuclear labelling oc curred in the BP compartment in all 93 mice irrespective of the occurrence of normal or atypical epithelium Thymidine labelled cells were present in the IM compartment in 10 of the 18 mice (56 per cent) with atypias grade I in 31 of the 35 mice (89 per cent) with atypias grade II and in all 19 mice with atypias grade III, but in none of the mice with normal epithelium Thymidine labelled cells occurred in the S compartment in 18 of the 19 mice (95 per cent) with atypias grade III and in 3 of the 35 mice (9 per cent) with atypias grade II The S compartment was autoradiographically negative in all mice presenting atypias grade I or normal epithe lium The difference between the proportion of mice with labelled cells in the S compart ment having normal grade I atypia and grade II atypia was significant ($p<0.01$) Signifi cant differences between grade II and grade III atypias ($p<0.001$) were also found

B) *Spayed mice treated with 3,4 benz pyrene* The autoradiographic patterns in the squamous epithelium of ovariectomized ani mals after local application of 3,4 benzpyrene are shown in Table 2 Three animals had normal epithelium whereas 4 animals showed atypia grade I Nuclear labelling was ob served in the BP compartment in all 7 ani mals Labelling in the IM compartment was observed also in two of 4 animals with atypia

TABLE 6 *Number of Mice with ³H Thymidine Labelled Cells in the Various Compartments of Normal and Atypical Epithelium During the Oestrous Cycle All Mice Treated with 3 4 Benzpyrene*

	Histology												
	Pro oestrous			Oestrous			Meta oestrous			Di oestrous			Total
	BP	IM	S	BP	IM	S	BP	IM	S	BP	IM	S	
Normal	3	–	–	13	–	–	3	–	–	2	–	–	21
Grade I	5	3	–	2	1	–	4	2	–	7	4	–	18
Grade II	10	9	–	9	7	–	7	6	1	4	4	1	30
Grade III	3	3	3	3	3	3	4	4	4	7	7	6	17
Total	21	15	3	27	11	3	18	12	5	20	15	7	86

BP denotes the basal parabasal compartment, IM the intermediate compartment and S the superficial compartment

grade I No labelled cells were observed in the S compartment

C) *Acetone treated controls* Labelled cells were observed in the BP compartment in all the 24 mice treated with acetone (Table 3) Labelling of the epithelial cells in the IM compartment was found in 5 out of 10 animals with atypia grade I No labelled cells were observed in the S compartment

Quantitative Determinations

The quantitative determinations of labelled cells in the various compartments in 3 treated mice with normal epithelium and in 11 mice with atypical epithelium are shown in Table 4 It is seen that the mean percentage of labelled cells in each compartment increased with increased epithelial atypia, except in the BP compartment in the case of atypias grade II and III

The percentage of labelled cells in 14 animals treated with 3,4 benzpyrene and presenting areas with epithelial buds as well as areas with smooth epithelial stroma border are presented in Table 5 It is seen that atypical epithelium with buds presented larger proportions of labelled cells than areas without epithelial buds the difference being statistically significant ($p<0\ 01$)

Thymidine Incorporation in the Various Phases of the Ovulatory Cycle

Table 6 shows the number of mice with labelled cells in the various compartments of normal and atypical epithelium during the oestrous cycle It is seen that the labelling of cells in the BP compartment occurred in all animals It should be pointed out that the normal epithelium in all three mice at meta oestrous was very thin and made up of few layers of cells The division of the normal epithelium at meta oestrous into 3 compartments was difficult and therefore not performed The atypical epithelium in mice at meta oestrous however, was usually thicker than the normal epithelium The division of the atypical epithelium into three compartments in mice otherwise presenting normal epithelium at meta oestrous was therefore performed From Table 6 it may be deduced that the relative proportions of mice with atypias and labelled cells in the IM and S compartments were apparently not greatly influenced by the various phases of the oestrous cycle

DISCUSSION

In the present investigation it was found that labelled cells (indicating DNA synthesis) were present in the deeper layers of the cervical epithelium of mice (i e near epithelium stroma border) in the normal as well as in atypical epithelium after iterated local application of 3,4 benzpyrene or acetone The presence of labelled cells in the IM and S compartments was observed in the atypical epi

thelium only Labelled cells in the S compartment occurred in the majority of the animals with severe atypias (grade III) and in a few of those with moderate atypias (grade II) As a rule, there was a good correlation between the degree of histological atypias and the pattern of thymidine incorporation Some of the cases presenting histological atypias grade II, however, diverged from the usual pattern and behaved autoradiographically as histolog cal grade III atypia (*Rubio* & *Lagerlof* 1973) Similar observations have been made in human cervices presenting moderate dysplasias of the epithelium (*Rubio* & *Lagerlof*, in press)

These observations suggest that despite cytoplasmic maturation towards the surface in atypias grade II, the nuclei in the S compartment of some mice retain the capacity of less mature cells and of undifferentiated cancer cells to replicate their DNA (*Oehlert* 1973) It is understood that in normal epithelium the cells with higher cytoplasmic maturation have lost the capacity of DNA synthesis (*Oehlert* 1973) It is thus conceivable that the presence of labelled nuclei in the S compartment in some mice with grade II atypias represents an alteration in the mechanism of DNA replication in those cells (*Davidson* 1963) One possibility is that those few cases of grade II atypias are biologically more active lesions although unrecorded as such by conventional histological criteria

Similar results in terms of qualitative labelling were obtained in intact and spayed animals exposed to the carcinogen This suggests that labelling patterns in the various epithelial alterations during carcinogenesis are not influenced to any appreciable degree by the ovarial function

The findings of quantitative determinations suggest that the proportion of DNA synthesizing cells in the atypical epithelium increases progressively with the degree of atypia Similar results have been obtained in the cervical epithelium of human cone specimens presenting various degrees of epithelial dysplasia or carcinoma *in situ* (*Rubio* & *Lagerlöf* in press)

Areas with atypical epithelium with buds presented a higher percentage of labelled cells than epithelium with similar atypias but devoid of buds in the same animals These findings indicate that the proportion of cells with active or recently completed DNA synthesis is larger in atypical epithelium with buds than in atypical epithelium with a smooth epithelium stroma border

In the light of these data it may be inferred that the areas with intra epithelial atypias with buds are biologically more 'active (i e in terms of proportion of nuclei in DNA replication) than areas with similar epithelial atypias but without buds The possible connection between the increased number of labelled cells in epithelial buds with atypia at the time of observation and the histological finding that micro invasive carcinoma originated at the tip of epithelial buds with atypias (*Rubio* & *Lagerlof* 1974) is at present being investigated in this laboratory

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The efficient technical assistance of *M Edenholm Y Kock I Kran* and *M Sveander* is gratefully acknowledged

This investigation was supported by a grant from the *Swedish Cancer Society*

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Acta path microbiol scand Sect A, 82, 483 486, 1974

THE SIGNIFICANCE OF MALLORY BODIES IN THE PROGRESSION OF FATTY LIVER INTO CIRRHOSIS

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Two groups of liver biopsies with fatty change, one with and one without Mallory bodies, are compared morphologically, and the corresponding groups of patients are compared biochemically and clinically Both the morphological and the biochemical activity are greater in the group with Mallory bodies and, as the morphological follow up shows a significantly more frequent development of cirrhosis in the group with Mallory bodies, there is good reason to believe that Mallory bodies and alcoholic hepatitis give rise to development of connective tissue in the parenchyma and are the common cause for development of cirrhosis in alcohol cs with livers exhibiting fatty change

In a previous paper by Christoffersen & Juhl (3) it was shown that among patients with steatosis, but without cirrhosis, the group with Mallory bodies had more liver cell necroses, a greater degree of inflammatory reaction more fibroses of the parenchyma, and a greater transaminase elevation than the group of patients without Mallory bodies

The work presented here is a folllow up investigation of the patients mentioned above with fatty change and Mallory bodies, and is aimed at obtaining an evaluation of the *possible significance of Mallory bodies for* the development of cirrhosis

MATERIAL AND METHODS

The material consists of two groups of patients all with liver biopsies showing fatty change as the chief morphological diagnosis Biopsies with cirrhosis suspicion of cirrhosis signs of cholestasis of chlorpromazine type or extrahepatic cholestasis, signs of viral hepatitis, malformations, neoplasm, and vascular disorders are excluded

Sixteen (*group 1*) contain Mallory bodies and have been collected as consecutive biopsies with fatty change and Mallory bodies from a total of 1,000 consecutive liver biopsies (1)

Group 2 comprises 67 consecutive patients whose liver biopsy exhibited fatty change without Mallory bodies (2)

The methods for technical procedures and assessment of biopsies have been described in a previous paper (3)

The clinical data have been based on particulars from the case record at the time of admission at which the primary biopsy was performed

At the time of biopsy the biochemical tests noted in Table 2 were performed

The consumption of alcohol has been registered Chronic alcoholism is defined as intake of more than 50 g ethanol/day for more than five years.

From 14 of the 16 patients comprising group 1 rebiopsies (12) or autopsy material (2) are available The average period of observation has been 44 months varying from one month to 83 months

Rebiopsies have been performed on 42 of the 67 patients comprising group 2 All the rebiopsies have been assessed according to the same criteria

Received 7 xii 73 Accepted 14 ii 74

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TABLE 1 *Frequency of a Number of Histological Qualities in the Groups with and without Mallory Bodies*

	Acidophil bodies		Degree of fatty infiltration			Lipogranulomas	
	0	+	+	+ +	+ + +	0	+
16 biopsies with Mallory bodies	3 (19%)	13 (81%)	2 (13%)	11 (69%)	3 (19%)	2 (13%)	14 (88%)
67 biopsies without Mallory bodies	55 (82%)	12 (18%)	34 (51%)	24 (36%)	9 (13%)	24 (36%)	43 (64%)
Differences (χ^2 test)	$p<0.001$		$p<0.02$			$0.20\ p>0.10$	

The figures indicate the number of biopsies in each subgroup of the seven qualities
The percentage which the number constitutes of each subgroup is given in parenthesis

Parenchymal inflammation		Parenchymal fibrosis		Portal fibrosis		Portal inflammation	
0	+	0	+	0	+	0	+
2 (13%)	14 (87%)	7 (44%)	9 (56%)	2 (13%)	14 (87%)	0 (0%)	16 (100%)
49 (73%)	18 (27%)	51 (76%)	16 (24%)	44 (66%)	23 (34%)	23 (34%)	44 (66%)
$p<0.001$		$p<0.05$		$p<0.01$		$p<0.05$	

as the primary biopsies The average period of observation has been 47 months varying from four months to 120 months

For the statistical evaluation the chi² test has been used for the histological parameters, while the t test has been used for the biochemical variables following logarithmic transformation of the values for alkaline phosphatase serum asparate transaminase and serum gamma globulin The limit for type I error (2α) has been placed at 0 05

RESULTS

Morphological Findings

Eleven (69 per cent) of the biopsies with Mallory bodies exhibit changes as seen in alcoholic hepatitis (necrosis of liver cells containing Mallory bodies with infiltration of neutrophils)

A comparison between the degree of a series of morphological changes in the biopsies in the two groups is shown in Table 1 There is a greater incidence of acidophil bodies and more pronounced parenchymal inflammation and parenchymal and portal fibroses in biopsies with Mallory bodies than in biopsies without Mallory bodies, while there is no decisive difference in the incidence of lipogranulomas, lytic liver cell necroses variation in size of nuclei and cells, portal inflammation, bile duct proliferation, cholestasis content of iron and lipofuscin or degree of fatty change

Biochemical and Clinical Findings

The clinical and laboratory parameters are entered in Table 2 There are no significant differences as regards sex, age or abuse of alcohol

Among the registered laboratory parameters serum asparate transaminase and BSP-retention are elevated in both groups but

TABLE 2 *The Distribution of the Clinical and Biochemical Parameters at the time of the Primary Biopsy in the two Groups of Patients with Fatty Change of the Liver 16 Patients with Mallory Bodies and 67 Patients without Mallory Bodies*

Variable (normal value)	Fatty liver with Mallory bodies (16 patients)		Fatty liver without Mallory bodies (67 patients)		Significance t test or chi² test
	Per cent	Mean ± s d	Per cent	Mean ± s d	
Men	56		70		n s*
Age (years)		60+11		56±12	n s
Alcoholism	75		72		n s
Alkaline phosphatase (<10 K A units)		10 6±3 8		10 0+4,5	n s §
Serum bilirubin (<1 0 mg/ml)		0 8±0 3		0 9±0 5	n s
Serum asparate transaminase (<1 7 mm ol/l/hr)		3 5±2 0		1 9±1 7	p<0 05§
Prothrombin Proconvertin (<60 per cent of normal)		81±19		87±14	n s
BSP retention (<5 per cent after 45 min)		19±9		14+9	p<0 05
Serum albumin (>4 4 g/100 ml)		3 44+1 06		4 45±0 74	p<0 01
Serum γ globulin (<1 1 g/100 ml)		1 0[illegible]±0 22		1 10±0 41	n s §

*Not significant i e p>0 05
§After logarithmic transformation

are significantly higher in the group with Mallory bodies Further, a significant reduction of serum albumin is found in this group The results of the other laboratory tests are within or just at the limit of the normal range

Follow up

Table 3 shows the distribution according to chief histological diagnosis of the repeat biopsies from 14 of the 16 patients in the group with Mallory bodies and from 42 of the 67 patients in the group without Mallory bodies

Three cases (21 per cent) from the group with Mallory bodies developed cirrhosis, and in two cases there is suspicion of cirrhosis Eight cases still show fatty change, and one case portal fibr[illegible]s without steatosis

In two of [illegible] es in the group without Mallory bo[illegible]rrhosis has developed, and most case[illegible] fatty change

The f[illegible]s from the group with Mallory bodies with cirrhosis or suspicion of cirrhosis all exhibit many Mallory bodies and alcoholic hepatitis in the primary biopsy and Mallory bodies were found in three of the repeat biopsies, and alcoholic hepatitis in one In one of the repeat biopsies still showing steatosis were alcoholic hepatitis or Mallory bodies demonstrated The observation time

TABLE 3 *The Distribution of Repeat Biopsies According to Histological Diagnosis in the Groups with and without Mallory Bodies*

Chief histological diagnosis	Repeat biopsies from 14 patients with Mallory bodies	Repeat biopsies from 42 patients without Mallory bodies
Cirrhosis	3	2
Suspicion of Cirrhosis	2	0
Fatty change	8	35
Liver with portal fibroses	1	5

for the three cases with cirrhosis are 8, 67 and 72 months, and for the cases with suspicion of cirrhosis one and 30 months

There are significantly more biopsies with cirrhosis or suspicion of cirrhosis in the group with Mallory bodies than in the group without ($p<0.02$) This applies, even when the case with short observation time is disregarded ($p<0.05$)

DISCUSSION

A grading of the histologic activity may be based on the number of disintegrating liver cells and the degree of inflammatory reaction in the parenchyma Greater activity is then observed in the group of biopsies with Mallory bodies than in that without This applies, even when necroses and neutrophils which are part of the alcoholic hepatitis and which are found in 69 per cent of the biopsies with Mallory bodies are disregarded

The biochemical changes are likewise more pronounced in the group with Mallory bodies where SGOT is significantly elevated

The above findings are in accordance with the follow up investigations as there are significantly more cases which develop cirrhosis in the group with Mallory bodies It thus seems probable that Mallory bodies in a fatty liver are a bad prognostic sign

Our investigation further shows that there is more pronounced parenchymal fibrosis with larger, often stellate, areas of connective tissue in biopsies with Mallory bodies than in the others As furthermore, the parenchymal fibroses in by far the greatest majority of cases are situated centrilobularly and with the same localization as Mallory bodies and alcoholic hepatitis, it is reasonable to believe that alcoholic hepatitis may give rise to development of connective tissue in the parenchyma and thereby play a significant role in the development of cirrhosis (4)

According to the available facts, it is thus very likely that Mallory bodies and alcoholic hepatitis are the common cause for development of cirrhosis in alcoholics with livers exhibiting fatty change

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Acta path microbiol scand Sect A 82, 487–494 1974

THE USE OF ANTITHYMOCYTIC SERUM IN THE TRANSPLANTATION OF HeLa-CELLS INTO RATS

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The use of rabbit anti rat thymocytic serum (ATS) as immunosuppressant was investigated in the heterotransplantation of HeLa cells into rats of different ages The transplantation was carried out by subcutaneous injection of HeLa cell suspension The first intraperitoneal ATS injection was given on the same day and the subsequent injections were given 2, 4, 7, and 10 days later On day 14, the rats were killed and the tumour growth was estimated by the size of the nodule and several histological criteria 0 005 ml ATS/g body weight given every time was observed to be sufficient to give a tumour growth in all animals aged less than 2 days A considerably bigger dose, 0 04 ml ATS/g body weight, did not further enhance the tumour growth or change the host reaction However, this dose caused a much higher mortality of animals 0 0005 ml ATS/g body weight did not cause adequate immuno suppression for transplantation of HeLa cells In experiments with older animals given 0 005 ml ATS/g body weight, the transplantation result in 7 day-old rats was comparable to that in younger rats, but clearly poorer results were obtained in 24 day-old rats It is concluded that adequate immunosuppression for the growth of HeLa cell tumours in rats 7 days old or younger was achieved by a dose of ATS sufficiently small for practical purposes

When human malignant tumours are to be transplanted into laboratory animals, several difficulties are found to be involved in immunosuppression With a view to resolving these problems it is of great advantage to use a model transplantation system where several different factors can be standardized and controlled In our laboratory we have used *heterotransplantation of HeLa cells into rats* for this purpose The work presented here describes experiments which were performed (1) to determine the optimal dose of anti thymocytic serum (ATS) to be applied to rats of different ages, and (2) to describe some morphological features of tumour and host tissue in animals under immunosuppressive treatment with ATS It has been found of value to have these points clarified before it is attempted to estimate the merits of ATS used alone or together with other immuno suppressants, for the establishment of a method as good as possible, by which human cancers can be transplanted into laboratory animals

Received 14 x 73 Accepted 19 ii 74

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MATERIALS AND METHODS

Laboratory animals The laboratory animals were from a stock of Wistar rats cross bread for 25

years at our Department Rats aged < 2, 7 or 24 days were used for the experiments They were not weaned from their mothers, who received normal laboratory animal diet and water *ad libitum*

The preparation of anti thymocytic serum (ATS) Thymocyte cell suspension was prepared from thymic gland of 1 month old rats which had been killed by ether The glands were washed in physiological saline and squeezed through a Millipore® stainless steel mesh (Stainless steel support screen cat no YY 22 142 64) into Medium 199 (Orion OY, Finland) The cells were washed three times with the tissue culture medium and, in between, centrifugated for 10 min at 250 g After washing, the number of cells in the suspension was adjusted to 10^9 cells/ml with the medium Rabbits were immunized by injecting 1 ml of this cell suspension into the ear vein, followed by another similar injection two weeks later, one week after the second injection, the rabbits were bled (*Levey & Medawar* 1966, *Jooste et al* 1968) The blood was allowed to clot, the serum was separated, and inactivated at 56° C for 30 min The serum was then absorbed by its own volume of rat erythrocytes for 1 hr at 37° C The ATS so obtained was stored at 20° C The serum (ATS 200) used in the following experiments was a pooled serum raised in six rabbits

In tests of the immunosuppressive activity of ATS 200 *in vivo*, 24 day old rats were immunized by 10^9 sheep red cells and ATS 200 or normal rabbit serum (NRS) was injected intraperitoneally, 0 005 ml/g body weight, on days 0 and 3 The immune response was measured on day 5 by rosette technique (*Bach & Antoine* 1968) and by titrating haemagglutinins In these experiments, ATS 200 caused 70 per cent reduction in the number of rosette forming spleen cells Haemagglutinin assays were carried out as follows 0 025 ml of two fold dilutions of rat sera and equal volumes of 2 per cent sheep red cells in saline were incubated in microtitre plates at 37° C for one hour and kept at 4° C overnight before reading the agglutinin titres macroscopically The mean $\log_2$ titre in ATS group was 2 0 + 1 4 (SD), compared to 4 3 + 0 9 in NRS control group

In vitro, the immunosuppressive activity of ATS 200 was tested according to *Bach & Antoine* (1968) Spleen cells were obtained from rats immunized by sheep red blood cells five days earlier no complement was used in the test Compared to NRS controls, ATS 200 inhibited the rosette formation by at least 25 per cent to the titre 1 8000

The transplantation of HeLa cells The HeLa cells were collected at the end of the logarithmic phase of growth and were then suspended in BME diploid medium (Grand Island Biological Company, Grand Island, N Y, U S A), containing 10 per cent calf serum Five million cells in a total volume of 0 1 ml were injected subcutaneously on both sides of the dorsal midline of the rat In preliminary experiments, 1, 5 and 20 million cells were given, and also two intramuscular injection sites (thigh and dorsum) were tested However, 5 million cells given subcutaneously in dorsum was found most appropriate The first ATS injection was given intra abdominally at the same time as the tumour cell transplantation, and subsequent injections were given on the 3rd, 5th 8th and 11th day The amount of ATS and normal rabbit serum given each time as well as the number of animals used in different experiments are given in Table 1 Special care was taken to prevent the leaking of ATS out of the abdominal cavity through the hole of the needle

The examination of the animals On day 14 the animals were killed by ether and the dorsal subcutis was carefully inspected Also the thorax and abdomen were opened and inspected macroscopi

TABLE 1 *Transplantation Results and Histological*

Group	ANIMALS No of survivors/total	Age (days)	Serum treatment (ml/g body weight)	Percentage of takes in survivors	No of tumour nodules	Volume * of tumour nodules
I	15/16	2	ATS 0 005	100	20	113±35
II	4/11	2	ATS 0 04	100	7	105±67
III	9/10	2	ATS 0 0005	33	3	8±4
IV	7/8	7	ATS 0 005	100	7	126±15
V	5/6	24	ATS 0 005	40	4	66±45
VI	12/12	2 7	NRS 0 005	0	0	0

Abbreviations ATS – rabbit anti rat thymocytic serum, NRS = normal rabbit serum

* Mean ± standard error of the mean The criteria used for estimation of the volume of the tumour nodules and for various scores of the tumours tissue or its connective tissue capsule, as well as the criteria for determining the final transplantation result, are described under Materials and Methods

cally for tumour nodules Whenever a lesion of any kind was observed, its dimensions, colour and lo cation in regard to the neighbouring tissues were recorded Then the whole lesion was fixed in 10 per cent neutral formalin, embedded in paraffin and prepared for routine histological examination The haematoxylin-eosin and van Gieson haematoxylin stainings were made of 6 μm tissue sections

The growth of HeLa-cell nodules in experimen tal animals was estimated as good, moderate or poor using the following criteria

"Volume of the nodule The volume was ob tained by multiplying the three dimensions (meas ured in millimeters) of the tumour nodule

Mitoses in cancer cells The number of mitoses were estimated subjectively as abundant (3), mod erate (2), few (1) or lacking (0)

Degeneration of tumour tissue Such tumour cells which had pycnotic nuclei, dense scanty cyto plasm and mostly showed no mitoses were regarded as degenerated cells (Figs 3 4) They formed smaller or larger areas in tumour nodules and the degree of degeneration in cancer tissue was re garded as abundant (3) if most of the cancer tissue was degenerated moderate (2) if about ¼ to ¾ was degenerated and mild (1) if less than ¼ was affected Cases marked by 0 are those where no degeneration was noticed

Leucocytes in tumour tissue These were pre dominantly polymorphonuclear leucocytes and their number was recorded from abundant (3) to lack ing (0)

The amount of the 'giant cell granuloma" These were granuloma containing multinucleated giant cells and mononuclear (histiocytic) cells often infiltrated by polymorphonuclear leucocytes The amount of granulomatous tissue was estimated in regard to preserved tumour tissue from abundant (3) to lacking (0)

Thickness of the connective tissue capsule around the cancer nodule This was subjectively graded as thick (2), thin (1) or lacking (0)

The amount of mononuclear cells and poly morphonuclear cells in or around the capsule Both were estimated subjectively from abundant (3) to lacking (0)

The final estimation of the tumour growth was carried out by summarizing the variants described above However, invert values were used in cases or degeneration inflammatory cell infiltration in tumour tissue or in its capsule, giant cell granuloma or they were applied to the thickness of the connective tissue capsule around the tumour Ac cording to this estimation, the results ranged from good (3) to poor (1) (cases where no cancer tissue was found were recorded by 0)

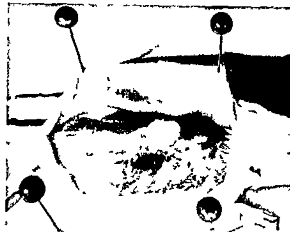
RESULTS

The results are summarized in Table 1

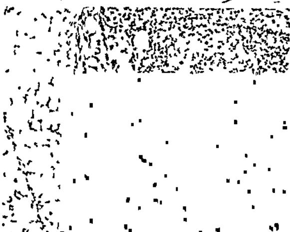
Group I (< 2 days old rats received 0 005 ml/g ATS) One to two tumour nodules were observed in all 15 animals The nodules varied in size from 1×1×1 to 10×9×6 mm and were most often about half the maximum size (Fig 1) In every nodule, carcinoma tis sue with abundant mitoses was found (Fig 2) In the middle of the tumour tissue, in 14 out of a total of 20 nodules, an area was found where the cancer cells were small somewhat loosely arranged and various ne-crobiotic changes were found in these, but mitoses were found infrequently (Figs 3, 4) These cells were classified as degenerated cancer cells Although the behaviour of

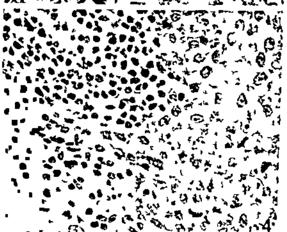
operties of Transplants of HeLa Cells in Rats

TUMOUR TISSUE Score* of				CONNECTIVE TISSUE CAPSULE Score of			FINAL RESULT
Mitoses	Degeneration	Leucocytes	Giant cell granuloma	Thickness	Mononuclear cells	Polymorphs	Score of
29±01	09±02	01±01	005±005	11+01	09±01	07±01	26±01
26±02	17±02	0	0	16±02	04±02	01±01	24±02
10±05	17±05	13+03	07 +05	13+04	27±03	13±03	13±05
25±02	10+0	08+05	07 ±03	15±02	12±02	11±02	24±07
27±02	17+04	13±06	17 ±02	19±01	12±02	15+02	10±0
–	–		–	–	–	–	0











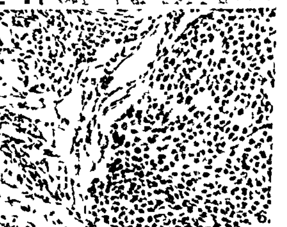


Fig 1 A tumour nodule in the dorsal midline attached to the underlying muscle

Fig 2 Solid tumour tissue where one of the several mitoses is atypical In the middle is seen a capillary × 400

Fig 3 Margin of a tumour which is demarcated from surrounding fat tissue by moderately thick layer of connective tissue Practically no inflammatory cells are seen around the tumour In tumour tissue a degenerated area is seen where the nuclei are pycnotic × 100

Fig 4 Degenerated and viable tumour tissue The cells in the former area (left) contain little cytoplasm and the nuclei are pycnotic Some disintegrating nuclei are seen In the viable tumour tissue mitoses are seen × 400

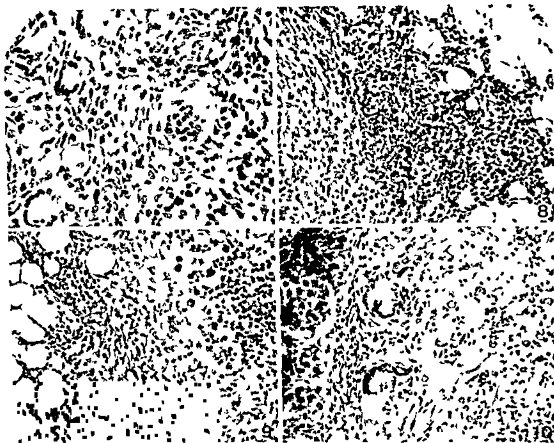


Fig 5 Tumour nodule surrounded by a rather thin connective tissue capsule A mild infiltration of mono nuclear cells is seen around the capsule × 250

Fig 6 No capsule is seen between the tumour t ssue and the surro inding fat ti si e The inflammatory cell infiltration is very scanty × 250

Fig 7 Giant cell granuloma containing multinucleated giant cells and mononuclear cells A few lympho cytes are seen at the periphery (right) One of the giant cells contains ingested material in its cytoplasm This nodule contained no cancer tissue × 400

Fig 8 The periphery of a nodule where only little cancer tissue was present Abundant inf ltration of mononuclear cells around the nodule is seen × 250

Fig 9 A cluster of degenerated tumour cells (r ght) surrounded by giant cell granulation and moderately abundant inf ltration of mononuclear cells × 400

Fig 10 Widely rejected tumour Several necrotic tumour cells with scattered leucocytes and two giant cells are seen The upper one of the giant cells contains a necrotic (tumour) cell in its cytoplasm Some cancer tissue is seen to the left × 400

growth of cancer parenchyme was distinctly medullar the cancer tissue in Group I as well as that in Groups II and IV contained some connective tissue stroma The stromal tissue elements however were often thin septae where only a few red cell containing capillar ies of small diameter could be seen (Fig 2) Extravasated erythrocytes could be seen in tissue spaces surrounded by cancer cells and especially among degenerated tumour cells

In all nodules connective tissue capsule of varying thickness was seen and it surrounded partly or completely the tumour (Figs 3 5 and 6) Very often inflammatory cells

around the carcinoma tissue were found In most cases, however, they were not abundant (Fig 5) Capillaries were often rather numerous in the connective tissue capsule around the tumour as well as in loose connective or adipose tissue around it

Group II (2 days old rats, 0 04 ml/g ATS) Only 4/11 rats in this group survived the experimental period, *viz* 14 days One to three tumour nodules were found in all of these 4 animals In one rat, one of the two nodules did not contain any cancer tissue but only giant cell granulation tissue while the other nodule in this rat was composed of viable tumour tissue In the three other cases, no distinct differences in tumour growth or host reaction could be observed as compared with the Group I

Group III (2 days old rats, 0 0005 ml/g ATS) Cancer nodules were found only in 3/9 surviving rats and in two of these, the transplantation result was regarded as poor In the third rat the final result was moderate In a further three rats, yellow nodules could be observed but no cancer tissue was found in these nodules, only giant cell granulation (Fig 7) The layer of connective tissue around the granuloma was not thicker than that surrounding the cancer in Groups I and II, but it contained a greater number of inflammatory cells (Figs 8, 9)

Group IV (7 days old rats, 0 005 ml/g ATS) Tumour nodules were observed in all of the 7 animals The inflammatory cells in the tumour tissue seemed to be more abundant than in Group I, but otherwise there were no apparent differences

Group V (24 day-old rats, 0 005 ml/g ATS) One to three yellow or yellowish nodules were found in all of the 5 animals No tumour growth was observed in three cases In two other cases, only poor tumour growth was observed The tumour nodules investigated microscopically contained giant cell granulation tissue, and the giant cells would often contain inclusions which seemed like necrotic tumour cells (Fig 10) The nodules were surrounded by a layer of connective tissue, perhaps a little thicker than that in other groups The inflammatory cell infiltration was more abundant than in groups I or II

Group VI (7 day-old or younger control rats, 0 005 ml/g NRS) No cancer growth was noticed in any of the 12 control animals In five rats, yellow nodules were found which, however, contained only giant cell granulation tissue surrounded by a layer of connective tissue not thicker than that in groups I–IV but infiltrated by numerous inflammatory cells No nodules were found in 7 other animals

Bone and Cartilage in Tumour Tissue

In 14/31 rats who developed cancer nodules, bone, osteoid tissue or cartilage was found All these rats were from groups I, II or IV Bone formation from cartilage was observed in some nodules Bone trabeculae were of various thickness, often surrounded by osteoblasts The medullary spaces were filled by loose connective tissue or cancer tissue

DISCUSSION

The HeLa cell line was selected for the study of the transplantation of human tumour xenografts because it is easily available It is difficult to estimate, however, how closely the HeLa-cell tumour immunologically is related with native human cancer because it may have lost some of its transplantation antigens during the long period of successive cell cultures Although ATS (or ALS) is especially suitable in difficult grafting conditions such as xenografts (*Medawar* 1969), its use has been rather limited in the transplantation of human tumours to animals

It is known that ALS or ATS raised in different animals of the same species may vary greatly as regards their immunosuppressive power ATS raised in different species of animals are also known to vary (*Jooste et al* 1968, *Antoine et al* 1970, *Bach et al* 1970) In our preliminary experiments (47 transplantations) with ATS raised in four rabbits the immunosuppressive activity of the sera differed On the other hand, if a pooled

ATS made up of sera from six rabbits was used consistent results would be obtained in experiments carried out under similar conditions This implies that the transplantation of HeLa cells into young rats can be used as an *in vivo* test for the immunosuppressive activity of different batches of ATS as already suggested by *Stanbridge & Perkins* (1969)

In the present study it was observed that successful transplantation of HeLa cells into newborn rats could be carried out using a dose of 0 005 ml ATS/g body weight Using this dose, good to moderate tumour growth was found in all the rats investigated thus indicating that this type of transplantation system can be used in further investigations Nearly 100 per cent of the HeLa cell transplants survived in mice given ALS (*Phillips & Ga et* 1967, 1968 *Stanbridge & Perkins* 1969) The period of observation in the present study was fourteen days rather a short time, and the dose may prove insufficient if tumours surviving for longer periods of time are to be studied However the dose of 0 04 ml ATS/g body weight was found to cause an inconvenient high mortality of the animals In preliminary experiments absorption of ATS once by rat red cells did not remove completely the anti erythrocyte antibodies

On the other hand, a smaller dose of ATS (0 0005 ml/g body weight) did not cause a sufficient immunosuppression as a tumour nodule surrounded by heavy infiltration of mononuclear cells was observed in only 3 out of 9 animals transplanted In addition histological examination of four rats showed that the yellow nodules did not contain any cancer tissue only a granuloma of giant cells mononucleated (histiocytic) cells and polymorphonuclear leucocytes The connective tissue capsule around the granuloma was heavily infiltrated by mononuclear cells

The nature of the giant cell granuloma remains somewhat obscure Its occurrence in the groups (III V and VI) where the immunosuppression apparently has been inadequate [illegible] tes that it may be an immune response *per se* It is known that cell mediated immunity is associated with many types of granulomatous reactions (*Epstein* 1967) However we cannot exclude the possibility that the granuloma has developed to scavenge the tumour cells already subjected to immune cytolysis This interpretation is favoured by an observation that giant cells sometimes contained ingested (tumour) cells

There are no distinct differences in tumour growth or host reaction in rats less than 2 days old and 7 day old rats who received the same dose of ATS (0 005 ml/g body weight) In both groups all the animals transplanted developed tumour growth The reverse applies to group V which consisted of 24 day old animals among which tumour growth was observed only in two of the five animals and even in these the result was regarded as poor since there were signs that rejection was *going on The remaining three rats developed* nodules which contained no cancer tissue. The histological findings in these cases indicate that complete rejection of the tumour tissue has taken place

The explanation of the observed age dependence in animals may be found in the development of the cell bound and humoral immunological mechanisms in rats aged 1 to 24 days Although onset of transplantation immunity in the rat (here the age at which allografts are not accepted for longer than 15 days) occurs 21 days post conception (*Steinmuller* 1961) and the earliest detectable antibody formation has been found 28 days post conception (*Hervey* 1966), there is experimental evidence that the developing immunological system in the rat shows reactions that are weaker in the early days of life than in the later this applies to the cell mediated as well as to the humoral immune responses (*Paterson* 1959 *Steinmuller* 1961, *Halliday* 1964)

The control rats never developed cancer nodules In five out of twelve [illegible] nodules were observed which contained only giant cell granuloma mixed with disintegrating leucocytes but no cancer tissue Around the granuloma there was a heavy infiltration

of mononuclear cells, indicating that immunological rejection has taken place

The thickness of the connective tissue capsule surrounding the nodules was not influenced by the immunosuppressive treatment Thus it seems that the capsule formation is not due to any immunological phenomenon, but presumably may be induced by a direct influence of tumour tissue on the host mesenchyme or connective tissue A glycoprotein like factor has been found in HeLa cells which, in cell culture, promoted the growth of human fibroblast-like cells (*Foley et al* 1963) However, as a thick connective tissue capsule around the tumour seemed to limit the tumour growth, the capsule formation was regarded as a negative quality when the transplantation result was estimated (Table 1)

Degenerative areas, often situated in the middle of the tumour nodule, may have been caused by poor vascularization of tumour tissue, although the possibility of some kind of direct immunological damage cannot be excluded *Phillips* & *Gazet* (1968) did not find any vascularization in HeLa cell tumour tissue in mice, nor any infiltrative growth In HeLa cell tumours of the rat there was a scanty stroma of fibrous tissue with a few capillaries

Bone, cartilage or osteoid tissue, which was seen in the cancer tissue in 14 out of the 31 rats with cancer nodules, develop probably as a result of some kind of induction by HeLa cells on rat connective tissue or mesenchyme This will be discussed in greater detail in a subsequent paper

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Acta path. microbiol scand Sect A, *82*, 495–498, 1974

EFFECTS OF SUPPLY AND WITHDRAWAL OF FLUORIDE

Experimental Studies on Growing and Adult Rabbits

7 Long term Observations on the Reversibility of Skeletal Fluorosis

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Fluoride supplement of 10 mg of fluoride per kg body weight per day given to growing rabbits during 14 weeks until skeletal growth had ceased resulted in the development of typical skeletal fluorosis Withdrawal of this supplement resulted in normalization of bone morphology after 52 weeks, i.e. during adulthood At that time, the bone fluoride concentration was nearly the same as that at the time of withdrawal (about 0 70 per cent of ash) Thus, bone remodelling and maturation did not result in any marked elimination of F from the bone mineral

The reversibility of skeletal fluorosis in cortical bone of femur and tibia has recently been studied chemically (*Rosenquist* 1973a, *Lemperg & Rosenquist* 1974) and morphologically (*Rosenquist* 1973b) A fluoride supplement of 10 mg per kg body weight per day during skeletal growth produced skeletal fluorosis with fluorotic bone areas localized mainly to the lateral part of the tibia Withdrawal of the fluoride supplement resulted in a gradual normalization of the bone morphology despite persistence of high fluoride concentrations in the bone However, 24 weeks' maintenance on a low fluoride diet was not sufficient to restore completely normal bone morphology The present investigation was therefore undertaken to determine whether there is further normalization of bone morphology and/or decrease of fluoride concentration in the bone mineral if fluoride intake is kept low for a period of one year

Received 29 i 74   Accepted 28 ii 74

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MATERIAL AND METHODS

24 rabbits aged 48–52 days were divided into one control (n = 10) group and one experimental group (n = 14) All animals were supplied with distilled water and fed a diet containing 2 p p m of fluoride (*Rosenquist* 1973a) The experimental animals received daily fluoride supplement of 10 mg per kg body weight per day for 14 weeks At the end of that time the fluoride supplement was withdrawn and the animals were followed for another 52 weeks

Sampling of bone powder for *fluoride determination* in bone ash (*Rosenquist* 1973a) and *morphological* studies by *microradiography, fluorescence microscopy* and *morphometry* were performed as previously described (*Rosenquist* 1973b) Fluorochromes were administered on the day of withdrawal of the fluoride supplement and subsequently at intervals of 24 weeks and 52 weeks Cortical bone from identical parts of the diaphyses was used for fluoride determination and for morphological studies, respectively The day of withdrawal of the fluoride supplement will be referred to as day 0

TABLE 1 *Fluoride Concentration in Cortical Bone of Fluoride Supplemented Rabbits Expressed as per cent of Ash M ± S D*

Obs time		0(21)		52(73)
Bones	n		n	
Femur	8	0 73±0 10	6	0 73±0 07
Tibia	8	0 76±0 10	6	0 69±0 05

Observation times in weeks calculated from the day of withdrawal of the fluoride supplement Actual age within brackets

RESULTS

Fluoride Analysis

The fluoride concentration expressed as per cent of bone ash was in the control group 0 02 per cent in both femur and tibia and did not change during the observation period In Table 1 it can be seen that the high fluoride concentrations in the experimental group did not alter with time

Morphological Observations

Tibia In the *control group* a periosteal circumferential bone lamella was present at day 0 Endosteally, minor areas with signs of resorption were seen in some animals After 52 weeks a circumferential lamella had also developed endosteally

In the *experimental group*, excessive periosteal bone had formed at the lateral part of the cortex at day 0 This fluorotic bone was characterized by thin, heavily fluorochrome labelled radiating trabeculae (Fig 1) It showed considerable porosity (Table 2) Resorption cavities were frequently seen No periosteal circumferential lamella was present and the endosteal margin was irregular due to extensive bone resorption

52 weeks after withdrawal, a normal morphology was found in the experimental group (Fig 2) The only remaining abnormality was a slight irregularity of the endosteal surface in 2 out of 6 specimens The amounts of compact bone labelled by the first two administrations of fluorochromes were small but fairly equal in the individual animals and of the same order of magnitude as the



Fig 1 Microradiograph of the lateral part of a tibial cross section from an experimental rabbit at day 0 (14 weeks on a fluoride supplement of 10 mg/kg/day) Excessive periosteal bone formation the central part of which is occupied by a large area mainly showing active resorption Black arrows indicate the approximate borderline between fluorotic and pre fluorotic bone × 20 alt × 40

amount of bone surfaces labelled by the last fluorochrome administration In many instances, fluorochrome from all three labellings could be seen at different distances from the bone surfaces The turnover of the compact bone thus appeared to have been low during the 52 weeks of observation

Femur At day 0, a periosteal circumferential lamella was present in the control group but not in the experimental group moreover, in the latter group increased bone resorption was observed in limited areas of the ventral endosteal surface In general the morphological alterations were slight After 52 weeks, circumferential lamellas were seen both endosteally and periosteally No differences between samples from the normal and experimental animals could be seen In general the fluorochrome labelling showed the same characteristics as in the tibia

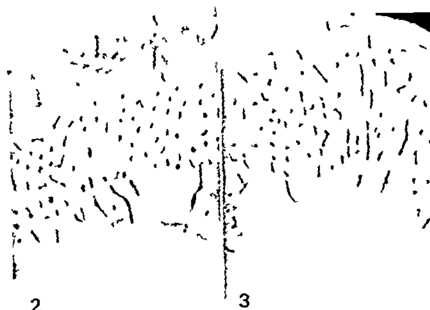


Fig 2 Microradiograph of the lateral part of a tibial cross section from an experimental animal 57 weeks after withdrawal of the fluoride supplement Note the presence of circumferential bone lamellae and the normal appearance of the bone as compared with Fig 3 × 40

Fig 3 Microradiograph of the lateral part of a tibial cross section from a control animal after 52 weeks of observation × 40

Morphometry

Table 2 shows how the initially high porosity of fluorotic bone was normalized

TABLE 2 *Relative porosity of Tibial Bone Expressed as Per Cent Non Mineralized Area per Surface Unit M ± S D*

Obs time		0(21)		52(73)
Group	n		n	
Control	5	3 6 ± 1 1	5	3 1 ± 1 0
Exptl	6	30 0 ± 3 5*	6	3 3 ± 1 2
* p<0 001				

In the experimental group periosteal trabecular areas were studied at day 0 and compact bone at 52 weeks, as in the control group

DISCUSSION

Withdrawal of a high fluoride supplement and intake of a low fluoride containing diet (? p p m F) resulted in partial normalization of bone fluorosis after 24 weeks (*Rosenquist* 1973b) The present study demonstrated that normalization of bone morphology continued further after 57 weeks bone fluorosis had disappeared and bone morphology was again normal This was mainly due to resorption of the periosteal exostoses but maturation of the fluorotic bone and remodelling had also contributed Two findings indicated that remodelling was not the main mechanism of normalization Firstly fluorochrome labelling indicated a low rate of turnover of the compact bone in the femur and in the part of the tibia remaining after resorption of the periosteal exostoses which is in agreement with previous observations (*Rosenquist* 1973b) Secondly the fluoride concentration was practically unchanged A significant decrease in the fluoride concentration would be expected if gross remodelling had taken place provided that excessive reutilization of fluoride *in loco* did not occur

Thus maturation of the spongy, fluorotic bone seemed to have been the main mechanism for normalization of the bone morphology The maturation process passed through an increasing bone mass per volume, by bone apposition and finally, brought about a normal compactnes Also the development of circumferential bone lamellae can be interpreted as part of maturation This small contribution of postfluorotic bone may account for the minimal decrease in the fluoride concentration observed in the tibia

Final normalization of the bone morphology occurred long after completion of skeletal growth, *viz* in adult animals This observation probably may be of practical value for the prognosis of skeletal fluorosis in man

Financial support was given by the *Swedish Medical Research Council* (projects 24 X 3925 and 17X 138)

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Acta path microbiol scand Sect A, 82, 499-506, 1974

SPLENIC METASTASES

Frequencies and Patterns

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Splenic metastases were found in 312 cases (7 1 per cent) in a uniformly examined post mortem series where it was possible to compare the frequency of metastases in different organs This frequency is comparable to, and in many cases higher than, the frequency of metastases in other organs with a corresponding risk of being the site of blood borne metastases The investigation does not suggest that the spleen has a greater antiblastic effect than other organs The metastases were divided into ten different types depending on their localization in the spleen and whether they were diagnosed macroscopically or microscopically or both Apart from type IV which probably consisted of continuous retrograde growth in trabecular lymph vessels and which cannot be regarded as true remote metastasis, the pattern of metastases suggests that splenic metastases are the result of haematogenous spread

On the basis of careful gross and microscopic examinations several authors have reported high frequencies of metastases in the spleen (e g *Deelman* 1918, *Yokohata* 1927, *Saphir* & *Parker* 1941 *Harman* & *Dacorso* 1948 and *Ondruš* 1949) The results of such investigations have, however, been questioned (*Walther* 1948, *Willis* 1952)

Modern text books usually state that secondary tumours in the spleen are rare Some authors ascribe this unsubstantiated assertion to an antiblastic effect of the spleen According to *Harman* & *Dacorso* (1948), the spleen is claimed to be "unfavourable to the development of secondary tumours" and they think that the statistical analysis has been customarily handled so as to favour such a conclusion 'The anticipated negative result is usually confirmed when examination of the organ is restricted to a single slice and a sceptical glance" (*Shaw Dunn* 1955)

Berge (1967) found metastases in the spleen at a frequency agreeing well with that found in the investigations referred to above He also described the frequency and distribution of secondaries set up by different primary tumours in a necropsy series examined in Malmo in the years 1958-1965 The investigation has now been expanded to embrace the 12 years 1958-1969

MATERIAL AND METHODS

From a hospital point of view, Malmo is a well defined region with one general hospital, one infirmary for chronic diseases and one mental hospital All three institutions are served by the Department of Pathology, Malmö General Hospital

During the above mentioned 12 year period the population of the town increased from about 220,000 to about 260,000 During these years, 26,048 individuals died and 16,294 (62 6 per cent) of the latter were examined post mortem (Of all the patients who died in hospital, 99 per cent were examined post mortem)

Received 28 ii 74 Accepted 28 ii 74

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TABLE 1 *Total Number, Number and Frequenc*

Site of primary tumour	Number of tumours			M .	
	m	f	tot	m	per cent
Prostate	1332	–	1332	299	22 4
Lung	569	178	747	505	88 8
Colon	344	361	705	185	53 8
Breast	2	693	695	2	100
Stomach	373	278	651	302	81 0
Kidney	218	138	356	100	45 9
Rectum	189	125	314	111	58 7
Pancreas	179	118	297	169	94 4
Liver	196	75	271	122	62 3
Ovary	–	253	253	–	
Biliary system	79	169	248	61	77 2
Urinary bladder + urethra	158	56	214	83	52 5
Uterine cervix	–	181	181	–	
Small intestine	102	57	159	27	26 5
Uterine body	–	111	111	–	
Oesophagus	76	34	110	58	76 2
Skin	54	44	98	31	57 4
Thyroid	25	42	67	19	76 0
Unknown	26	39	65	26	100
Renal pelvis + ureter	16	18	34	12	75 0
Larynx	30	0	30	13	43 3
Vulva	–	23	23	–	
Appendix	11	8	19	5	45 5
Oral cavity	12	6	18	8	66 7
Testes	18	–	18	15	83 3
Eye	10	6	16	9	90 0
Hypopharynx	10	4	14	5	50 0
Lip	11	3	14	3	27 3
Anus	3	10	13	2	66 7
Uterus unspec		13	13	–	
Maxillary sinus	8	4	12	4	50 0
Salivary gland	3	7	10	1	33 3
Tongue	7	3	10	2	28 6
Penis	9	–	9	2	22 2
Epipharynx	7	1	8	7	100
Tube	–	7	7	–	
Vagina	–	7	7	–	
Adrenal	3	2	5	3	100
Tonsil	2	2	4	2	100
Cardia	2	1	3	2	100
Sweat gland	1	1	2	0	
Nasal cavity	0	1	1	0	
Trachea	0	1	1	–	
Total	4085	3080	7165	2195	53 7

Metastasizing Tumours and Number of Organs with Metastases

tumours				Number of organs to which metastasizing tumours had spread		
				Lymph node groups	Other organs	Other organs excl serous cavities
f	per cent	tot	per cent	tot	tot	tot
–		299	22 4	2 8	2 3	2 0
139	78 1	644	86 2	2 5	3 1	2 7
223	61.8	408	57 9	1 7	2 3	1 8
539	77 8	541	77 8	4 3	3 8	3 1
231	83 1	533	81 9	2 7	2 1	1 4
63	45 7	163	45 8	1 3	3 1	2 6
79	63 2	190	60 5	1 7	2 4	2 0
108	91 5	277	93 3	2 0	2 5	1 8
47	62 7	169	62 4	1 9	2 0	1 6
217	85 8	217	85 8	3 4	2 7	1 5
147	87 0	208	83 9	2 2	1 8	1 3
30	53 6	113	52 8	2 3	2 3	1 9
109	60 2	109	60 2	2 5	2 4	1 9
21	36 8	48	30 2	1 2	0 8	0 7
49	44 1	49	44 1	1 8	2 4	1 7
25	73 5	83	75 5	1 9	1 8	1.5
28	63 6	59	60 2	3 2	8 1	7 2
23	54 8	42	62 7	1 9	2 9	2 6
39	100	65	100	2 5	3 6	2 9
17	94 5	29	85 3	1 6	2 8	2 6
–		13	43 3	1 8	2 4	1 9
17	73 9	17	73 9	2 4	3 9	3 0
2	25 0	7	36 8	1 3	1 9	0 7
3	50 0	11	61 1	2 1	2 1	1 6
–		15	83 3	2 3	4 3	3 5
6	100	15	93 8	2 3	8 7	7 7
2	50 0	7	50 0	1 9	1 4	1 1
2	66 7	5	35 7	0 8	0 8	0 8
7	70 0	9	69 2	2 2	1 9	1 6
11	84 6	11	84 6	2 9	3 4	2 6
1	25 0	5	41 7	1 6	1 6	1 2
4	57 1	5	50 0	2 6	4 0	3 8
2	66 7	4	40 0	2 8	1 3	1 0
		2	22 2	1 5	1 0	0 5
1	100	8	100	1 9	1 6	1 5
6	85 7	6	85 7	2 2	1 8	0 7
4	57 1	4	57 1	2 3	2 0	1 5
2	100	5	100	1 4	1 8	1 8
1	50 0	3	75 0	2 3	0 3	0 3
1	100	3	100	1 0	3 0	2 0
1	100	1	50 0	4 0	6 0	3 0
1	100	1	100	0	8 0	7 0
1	100	1	100	1 0	0	0
2209	71 7	4404	61 5	2 5	2 7	2 2

In 7,246 subjects (44 5 per cent of those examined post mortem), 8,214 primary malignant tumours were found Among these were 7,165 carcinomas The distribution of the primary malignant, epithelial tumours is given in Table 1

The table shows not only the total number of tumours, but also the numbers and percentages of metastasizing tumours as well as the mean number of organs or organic systems with metastases

As in *Walther's* (1948) and *Willis'* (1952) investigations, involvement of the lymph nodes was ascribed to lymphogenous spread, while metastases in other organs, except the large serous cavities, were regarded as the result of haematogenous spread The following lesions were regarded as metastases gross or microscopic tumour foci, separated from the primary tumour, with signs of infiltration, and intravascular tumour foci with *signs of growth*

The necropsies were performed in a uniform way during the investigation The methods have been described in detail elsewhere (*Berge* 1967)

In calculations of the frequency of metastasis only metastasizing tumours were considered The pattern of the metastases was judged essentially according to the principles of *Marymont* & *Gross* (1963) The various types are as follows

Microscopically Diagnosed Metastases

I a Cases with metastases in venous sinusoids without infiltration or involvement of the parenchyma

I b Cases of diffuse so called leucotic metastasis

II a Cases where metastases had replaced part of the parenchyma in the red pulp

II b Cases with one or more metastatic foci that had replaced parts of both the red and the white pulp

III Cases where parts of the white pulp had been replaced by metastases

IV Cases with tumour growth in the trabecular lymphatics

V Cases with involvement of several different elements of the spleen (I a + II b, I a + IV, III + IV)

Macroscopically Diagnosed Metastases

VI Cases with one or more nodular metastases surrounded by preserved splenic parenchyma

VII Cases where the parenchyma of the spleen had been more or less completely replaced by tumour tissue

Cases of Macroscopically Diagnosed Metastases where Histological Examination Revealed also Microscopic Foci

VIII Embraces the combinations I a + VI, I b + VI, II a + VI, III + VI and IV + VI

The data were treated statistically using the X^2 test $p<0.02$ was regarded as significant

RESULTS

Of the 7,165 carcinomas, 4,404 (61 5 per cent) were found to have spread to one or more organs The frequency of secondaries set up by all metastasizing carcinomas in different organs is given in Table 2

Metastases were found in the spleen in 312 cases (7 1 per cent) Their distribution among different primary tumours is given in *Table 3*

The number and frequency of different types of metastases are given in Table 4, together with data published by Marymont & Gross (1963)

DISCUSSION

Comparison of different series is often difficult because of differences in the composition of the materials, in the methods used at necropsy, and the principles of histological examination as well as in the definition of the term metastasis The basic material is often incompletely described and the same applies to the age and sex distribution For instance, routine histological examination of the prostate will often reveal latent carcinoma without metastases This applies in particular to series comprising many old subjects The total frequency of metastases will therefore be relatively low Series from special departments for the care of cancer often include advanced cases and thus the frequency of metastases will be relatively high The effect of such differences can be partly diminished if nothing but tumours that have metastasized are considered But information on this point is often missing

Most investigators try to avoid the inclusion of growth by extension as metastasis but

TABLE 2 *Incidence of Metastases in Entire Material*

		Number	%
1	Lymph nodes	3824	86 8
2	Liver	2202	52 0*
3	Lungs	2097	47 6
4	Skeleton	1425	32 4
5	Peritoneum	1339	30 4
6	Pleura	980	22 3
7	Adrenals	823	18 7
8	Kidneys	371	8 4
9	Brain	342	7 8
10	Spleen	312	7 1
11	Pericardium	248	5 6
12	Skin	240	5 4
13	Ovary	221	5 0
14	Myocardium	192	4 4
15	Thyroid	191	4 3
16	Pancreas	178	4 0
17	Meninges	149	3 4
18	Small intestine	118	2 7
19	Breast	88	2 0
20	Hypophysis	87	2 0
21	Stomach	85	1 9
22	Colon	62	1 4
23	Muscle	46	1 0
24	Biliary system	40	0 9
25	Uterine body	33	0 7
26	Prostate	29	0 7
	Renal pelvis + ureter	29	0 7
28	Urinary bladder + urethra	28	0 6
29	Testes	16	0 4
30	Oesophagus	13	0 3
31	Tongue	9	0 2
	Vagina	9	0 2
33	Diaphragm	8	0 2
34	Salivary gland	7	0 2
	Appendix	7	0 2
36	Parathyroid	6	0 1
37	Tonsil	5	0 1
38	Eye	3	0 1
	Oral cavity	3	0 1
40	Trachea	2	0 05
	Penis	2	0 05
	Tube	2	0 05
	Hypopharynx	2	0 05
44	Maxillary sinus	1	0 02
	Larynx	1	0 02

* Only extrahepatic carcinomas

the interpretation can be more or less liberal The large differences in frequency in different materials (for literature see *Berge* 1967) are probably not true but due to the above mentioned factors

TABLE 3 *Number and Frequency of Metastases to the Spleen from Different Primary Tumours*

Site of primary tumour	No	%
Breast	65	(12 0)
Lung	57	(8 9)
Ovary	26	(12 0)
Stomach	22	(4 1)
Skin	20	(33 9)
Prostate	18	(6 0)
Colon	16	(4 4)
Liver	14	(8 3)
Pancreas	13	(4 7)
Uterine cervix	8	(7 3)
Urinary bladder + urethra	7	(6 2)
Thyroid	6	(14 3)
Unknown	6	(9 2)
Kidney	5	(3 1)
Uterine body	4	(8 2)
Vulva	3	(17 6)
Rectum	3	(1 6)
Epipharynx	2	(25 0)
Anus	2	(22 2)
Small intestine	2	(4 2)
Oesophagus	2	(2 4)
Cardia	1	(33 3)
Adrenal	1	(20 0)
Salivary gland	1	(20 0)
Hypopharynx	1	(14 3)
Uterus unspecified	1	(9 1)
Larynx	1	(7 7)
Eye	1	(6 7)
Renal pelvis + ureter	1	(3 4)
Biliary system	1	(0 5)

If the rare types of primary tumours be ignored it will be seen (Tables 1 and 3) that splenic metastases are, as a rule, set up more often by tumours known to have a strong tendency to metastasize This applies, above all to tumours of the breasts, ovaries bronchi and skin (melanoma) and of these especially those that have metastasized to several organs via the blood stream This is well documented in the literature While tumours in these organs in the Malmo series were responsible for 53 8 per cent of all secondary tumours in the spleen they constituted only 33 2 per cent of the entire number of metastasizing tumours

Table 2 shows that the spleen is the tenth from the top among the most frequent sites of secondary tumours This agrees well with

TABLE 4 *The Type of Metastatic Cancer in the Spleen*

Appearance and localization of metastases in the spleen	Marymont & Gross (1963) No of cases	Marymont & Gross (1963) Percentage of total cases	Present series No of cases m	f	tot	Present series Percentage of total cases
Microscopic tumour only	31	33.3	50	49	99	31.7
I a – in venous sinusoids	11 (I a + I b)	11.8 (I a + I b)	14	8	22	7.1 } 13.5
I b – diffuse, leucotic type			13	7	20	6.4 }
II a – in red pulp as microscopic nodules	6	6.5	1	6	7	2.2
II b – in red as well as in white pulp	0	–	3	6	9	2.9
III – in white pulp as microscopic nodules	1	1.1	4	2	6	1.9
IV – in trabecular lymph vessels	1	1.1	12	16	28	9.0
V – in several different elements of spleen	12	12.8	3	4	7	2.2
Macroscopic tumour only	62	66.7	85	104	189	60.6
VI – nodular type	54	58.1	84	100	184	59.0
VII – diffuse type	8	8.6	1	4	5	1.6
Micr + Macr VIII	0	–	9	15	24	7.7

(type V = I a + IV – 2 cases, I a + II b – 3 cases III + IV – 2 cases.)
(type VIII = I a + VI – 18 cases, I b + VI – 2 cases, II a + VI – 1 case, III + VI – 1 case, IV + VI – 2 cases.)

earlier findings (*Buday* 1910, *Kitain* 1922, *Mc Whorter* & *Cloud* 1930, *Walther* 1948, *Galluzzi* & *Payne* 1955, *Orcel* & *Guyet* 1958, *Nash* & *Sampson* 1966).

Splenic metastases are rare compared with metastases in the lymph nodes, liver, lungs and skeleton, but the table gives a large number of organs where metastases are much rarer than in the spleen. Yet these organs are not believed to have any antiblastic activity. The reason why the spleen has this special position is presumably that given by *di Biasi* (1926), namely its histological similarity to lymph nodes where metastases are very common. It should, however, be borne in mind that the spleen is a lymphoid organ differing from the lymph nodes in its relation to the general circulation. Tumour cells must enter the blood stream before they can set up secondaries in the spleen. This holds true also for most sites of metastases where the frequency of secondaries is lower than in the spleen, but only for 4 of the organs where metastases are more common. It does not apply to the lymph nodes, lungs, peritoneum or pleura or the liver in cases where the tumours are drained by the vena portae. But in most tumours it does hold true for the skeleton, adrenals and kidneys as well as for the liver in subjects with tumours not drained by the vena portae. (The frequency, however, is higher (about 45 per cent) also for tumours not drained by the vena portae.) Compared with the kidney and the brain there is no significant difference. This means that only three organs contain blood borne metastases significantly more often than the spleen ($p<0.001$). There is thus no reason to ascribe any antiblastic activity to the spleen, and the assertion that splenic metastases are rare is wrong.

In series where interest has apparently not been focused especially on the spleen, the frequency of histologically diagnosed metastases varies between 0 per cent and 36 per cent of the total number of splenic metastases

(mean 22 2 per cent) In special investigations the frequency varies between 11 1 per cent and 100 per cent (mean 51 7 per cent) (for literature see *Berge* 1967) These high frequencies of microscopically diagnosed splenic metastases, however, have been demonstrated in selected cases of advanced cancer In such series, the frequencies will vary with the degree of selection which in turn depends on the definition of the term "wide spread malignancy" In *Berge*'s (1967) special investigation microscopic splenic metastases were thus found in 50 per cent of all subjects who had metastases in five or more organs or organic systems. In individuals with splenic metastases coexisting secondaries were found in, on the average, 4 5 lymph node groups and in 5 5 other organs (excluding serous cavities) In individuals without splenic metastases in the same series the corresponding figures were 2 5 and 1 9, respectively The largest difference was thus found in respect of haematogenous spread

These high frequencies were thought to suggest that the spleen has an inhibitory effect and that it can prevent microscopic metastases from developing further (*Muir* 1964) But such special investigations cannot be used as an argument unless also other organs are examined in the same way This was done by *Berge* (1967) in 100 consecutive cases of cancer, where 10 sections from each spleen pancreas, adrenal and kidney were examined microscopically These organs were selected because of their anatomic localization and a comparable risk of being involved by blood borne tumour emboli It was found that the frequency of microscopically demonstrable metastases in these four organs agreed well with the total frequency of metastases in these organs in all the metastasizing cancers

As for the metastatic pattern in the spleen the literature is scanty Many authors make a distinction between gross and microscopically demonstrated metastases Such a difference is essential since the route of spread of a tumour can only be expected to be judged from microscopically demonstrable metastases The first detailed study of the metastatic pattern was that by *Marymont & Gross* (1963) They recognized seven types of metastases, the first five of which were metastases diagnosed microscopically, but not seen at gross examination The small modifications made in the present investigation consist in the fact that their group I has been divided into two subgroups (Ia and Ib) These subgroups are essentially similar, but group Ia includes only one or a few smaller groups of malignant cells in the sinusoids, while type Ib has such cells in practically every field of vision The malignant cells are, as a rule, not adherent to the vessel wall This is the reason why this type of metastasis is called leucotic It is essential to distinguish this from the gross diffuse growth with tissue destruction (type VII) This type of diffuse, leucotic metastasis usually occurs in cases of poorly differentiated carcinoma This type of metastasis is not specific for the spleen, and in many cases, but not in all there was diffuse leucotic metastasis also in the liver and in the bone marrow It has been suggested that this diffuse, leucotic spread of the tumour is a terminal phenomenon (*Warren & Davis* 1934) This, however, appears less likely because if it were, one would expect to find the same picture in all the organs examined

Type IV is probably a continuous, retrograde growth in the lymph vessels and was demonstrated, above all, in carcinoma of adjacent organs (stomach and pancreas) It is probably not a real metastasis The reason why this type was included was, however, that it is included in other investigations and secondly, in the present study it was included also in organs other than the spleen It was thus necessary for comparison both in the present and in other series

The different types of metastases have been described in detail by *Marymont & Gross* (1963) and *Berge* (1967) In both these investigations it was concluded that the metastatic pattern suggested that the tumour spread to the spleen mainly via the bloodstream. *Sträuli* (1969) arrived at the same

conclusion This opinion is supported further by the fact that splenic metastases are found mainly in cases of tumour that has spread haematogenously to various organs or organic systems

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Valuable assistance was given by *Arne Sundstrom* at the Computer Central, University of Lund

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EXTRAMEDULLARY HAEMOPOIESIS AND METASTASES IN THE SPLEEN

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Among 284 cases of haematogenous splenic metastases, haemopoiesis was demonstrated in the spleen in 38 0 per cent Blood formation was found in 17 4 per cent of the cases without, and in 47 9 per cent of the cases with skeletal metastases ($p<0.001$) Among the cases with skeletal metastases, extramedullary blood formation was demonstrated in 68 7 per cent of those presenting involvement of all the vertebral bodies compared with 25 8 per cent of the remainder ($p<0.001$) The latter frequency (25 8 per cent) did not differ significantly from that (17 4 per cent) found among cases without skeletal metastases The occurrence of skeletal metastases therefore appears to be of little importance in cases without extensive destruction of the bone marrow Among cases with metastases in *all* the vertebral bodies, the frequency of extramedullary blood formation did not vary significantly with the type of metastases Among other cases with or without skeletal metastases, extra medullary blood formation was more common among cases with sinusoidal metastases This finding must, however, be judged with caution

In the foetus the spleen takes part in blood formation Later in life the spleen may again begin to form blood, but our knowledge of the factors stimulating such activity is still incomplete The activity is generally regarded as a compensatory mechanism in anaemia, especially in medullary insufficiency due to leukaemia, myelofibrosis or metastases

But myeloid metaplasia is not always found in these conditions, besides which it may occur in the absence of any of these diseases This implies the existence of other factors of significance in the stimulation of blood formation in the spleen

Opinions differ as regards the existence of a direct relation between myeloid metaplasia and metastases in the spleen *di Biasi* (1926) and *Lubarsch* (1927) found no tendency to myeloid metaplasia in patients with secondaries in the spleen *Blaustein* (1963) claimed that splenic metastases have no effect on the haemopoiesis *Hirschfeld* (1920), however, found that myeloid metaplasia was common in the vicinity of metastases According to *Gross & Marymont* (1963), there is a correlation between haemopoiesis and the pattern of metastases in the spleen *Berge* (1967) found that both the frequency and the extent of skeletal metastases varied with the metastatic pattern in the spleen and that cases comparable in respect to skeletal metastases showed largely the same frequency of myeloid metaplasia, irrespective of the metastatic pattern

MATERIAL AND METHODS

The material and the pattern of metastases have been described previously (*Berge* 1974) All 312 cases with splenic metastasis are given in Table I where they are grouped according to the metasta-

Received 28 ii 74 Accepted 28 ii 74

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TABLE 1 *Extramedullary Blood Formation and Skeletal Metastases In Subjects with Different Patterns of Metastases in Spleen*

Pattern of metastases	Number	Extramedullary blood formation	Skeletal metastases
Growth in trabecular lymph vessels (IV)	28	5 (17 9 %)	7 (25 0 %)
Growth in trabecular lymph vessels in combination with other types (I a + IV, III + IV, IV + VI)	6	3 (50 0 %)	2 (33 3 %)
Nodular (II a, II b, III, VI, II a + VI, III + VI)	208	57 (27 4 %)	128 (61 3 %)
Sinusoidal permeation only (I a, I b)	42	29 (69 0 %)	36 (85 7 %)
Sinusoidal permeation and nodular (I a + II b, I a + VI, I b + VI)	23	17 (73 9 %)	21 (91 3 %)
Diffuse (gross) (VII)	5	2 (40 0 %)	5(100 0 %)
Total	312	113 (36 2 %)	199 (63 8 %)

tic pattern, irrespective of whether they were diagnosed macroscopically and/or microscopically

In comparative investigations of different types of metastases and extramedullary blood formation 28 cases with secondaries only in trabecular lymph vessels were not included because in these cases it was most probably a question of retrograde growth by extension rather than of true metastasis More over, there was no contact between the metastases and the splenic parenchyma The six cases with growth in the lymph vessels as well as in the parenchyma were classified according to the latter site of metastases No continuity was found anywhere between tumour growth in the lymph vessels and the parenchyma

The group 'nodular + sinusoidal is missing in *Gross & Marymont s* (1963) material It was, however, included in the present material because it constituted a relatively large group and, secondly, it appeared to represent haematogenous spread, which was probably the case also in the other groups

In order to get the materials as comparable as possible in the evaluation of myeloid metaplasia, it was decided to use the same criteria as *Gross & Marymont* (1963) only spleens in which several typical aggregates of such cells could be easily identified were regarded as manifesting hematopoietic activity

It was not possible to assess the extent of medullary destruction in the entire skeleton, since routine examination included only opening of the vertebral column In addition histological examination was generally confined to demonstrated gross lesions

Neither was it possible to decide to what extent involvement of the vertebral column was representative of the entire skeleton The part of the skeleton, most often involved by metastases, however, is the spinal column (*Walther* 1948, *Willis* 1952) 'Indeed, if there are metastases in the skeleton at all, they will almost certainly be present in the vertebral collum whether other skeletal areas are also involved or not (*Jaffe* 1958)

In adults the vertebral column contains the major part of the active bone marrow, and in those cases where at most a few of the vertebral bodies contain metastases there is no reason to expect extensive destruction of the bone marrow Such destruction may be expected mainly in cases with secondaries in all the vertebral bodies

The destruction of the bone marrow was therefore assessed from the extent of involvement of the spine The cases were divided into three groups

I Metastases in all vertebral bodies

II Metastases in one or more vertebral bodies,

III No metastases in any of the vertebral bodies

The data were treated statistically using the X^2 test $p < 0.02$ was regarded as significant

RESULTS

The results are given in Table 2

The table gives the frequency and number of cases with skeletal metastases among the various types of metastases (except for type IV) It also gives the number and frequency of extramedullary haemopoiesis in cases with respectively without skeletal metastases

Extramedullary haemopoiesis was demonstrated in 38 0 per cent of the 284 cases with splenic metastasis Extramedullary haemopoiesis was found in 17 4 per cent of the cases

TABLE 2 *Extramedullary Blood Formation in Subjects with Different Types of Metastases in Spleen*

Type of metastases	Number of subjects	Subjects with skeletal metastases	Number and percentage of subjects with extra medullary blood formation among subjects		
			with skeletal metastases	without skeletal metastases	Total
I a	22	18 (81 8 %)	11 (61 1 %)	1 (25 0 %)	12 (54 6 %)
I b	20	18 (90 0 %)	15 (83 3 %)	2 (100 %)	17 (85 0 %)
II a	7	5 (71 4 %)	1 (20 0 %)	2 (100 %)	3 (42 9 %)
II b	9	8 (88 9 %)	5 (62 5 %)	– –	5 (55 6 %)
III	6	5 (83 3 %)	3 (60 0 %)	– –	3 (50 0 %)
V	7	3 (42 9 %)	1 (33 3 %)	1 (25 0 %)	2 (28 6 %)
VI	184	108 (58 7 %)	36 (33 3 %)	9 (11 8 %)	45 (24 5 %)
VII	5	5 (100 %)	2 (40 0 %)	– –	2 (40 0 %)
VIII	24	22 (91 7 %)	18 (81 8 %)	1 (50 0 %)	19 (79 2 %)
Total	284	192 (67 6 %)	92/192 (47 9 %)	16/92 (17 4 %)	108/284 (38 0 %)

without, and in 47 9 per cent of the cases with skeletal metastases The difference was significant (p<0 001) In order to study the relation between the metastatic pattern and haemopoiesis in the spleen more closely, the metastases were distributed, mainly according to *Gross & Marymont* (1963), among four large groups (nodular metastases, metastases confined to sinusoids, nodular metastases + metastases confined to sinusoids, diffuse (macroscopic)) The distribution is given in Table 3

The table gives the number and frequency of extramedullary blood formation and skeletal metastases in the cases with various types of metastases Columns I and II give the number and frequency of subjects with extramedullary haemopoiesis and skeletal meta stases, respectively column III, extramedullary blood formation in subjects with skeletal metastases, column IV, gives the figures for subjects with metastases in all vertebral bodies and column V gives the figures for sple nic haemopoiesis among these cases Column VI gives the figures for extramedullary blood formation in other individuals with skeletal metastases while column VIII gives the corresponding figures for extramedullary haemopoiesis in subjects without skeletal metastases

Gross & Marymont's (1963) data which were used for comparison are given in Table 4

DISCUSSION

Gross & Marymont (1963) found that in cases with splenic metastases confined to the sinusoids myeloid metaplasia in the spleen was more than four times as common as in those with nodular metastases The frequencies of skeletal metastases in the two groups were 82 per cent and 56 per cent, respectively But they did not assess the severity of medullary destruction In a control series consisting of 50 cases of metastasising carcinoma without splenic metastases they found myeloid metaplasia in the spleen in 14 per cent They thought that this frequency was "roughly comparable" with the 21 per cent in individuals with nodular metastases

Most of their patients had moderate to grave anaemia They therefore thought that anaemia could not explain the differences Neither did they feel that the difference in frequency of skeletal metastasis (82 per cent and 56 per cent, respectively) was sufficient to explain the large differences in the frequency of myeloid metaplasia They therefore assumed a causal relation between myeloid metaplasia and metastasis localized to the sinusoids They made no definite conclu-

TABLE 3 *Extramedullary Blood Formation and Skeletal Metastases in Subjects with Different Patterns of Metastases*

Pattern of metastases	I Extramedullary blood formation	II Skeletal *metastases*	III Extramedullary blood formation among subjects with skeletal metastases	IV Carcinomatous involvement of all vertebrae	V Extramedullary blood formation among subjects with carcinomatous involvement of all vertebrae	VI Extramedullary blood formation among other subjects *with* skeletal metastases	VII Extramedullary blood formation among subjects *without* skeletal metastases
Nodular	(60/212) 28 3 %	(130/212) 61 3 %	(48/130) 36 9 %	(52/130) 40 0 %	(33/52) 63 5 %	(15/78) 19 2 %	(12/82) 14 6 %
Sinusoidal	(29/44) 65 9 %	(36/44) 81 8 %	(26/36) 72 2 %	(24/36) 66 7 %	(19/24) 79 2 %	(7/12) 58 3 %	(3/8) 37 5 %
Sinusoidal + Nodular	(17/23) 73 9 %	(21/23) 91 3 %	(16/21) 76 2 %	(18/21) 85 7 %	(14/18) 77 8 %	(2/3) 66 7 %	(1/2) 50 0 %
Diffuse (gross)	(2/5) 40 0 %	(5/5) 100 %	(2/5) 40 0 %	(5/5) 100 %	(2/5) 40 0 %	—	—
Total	(108/284) 38 0 %	(192/284) 67 6 %	(92/192) 47 9 %	(99/192) 51 6 %	(68/99) 68 7 %	(24/93) 25 8 %	(16/92) 17 4 %

Differences between frequencies in Table 3 as measured with the Y^2 *test (only significant differences are given)*

I Extramedullary blood formation

II Skeletal metastases

III Extramedullary *blood formation* among subjects with skeletal metastases

IV Carcinomatous involvement of all vertebrae

VI Extramedullary blood formation among other subjects with skeletal *metastases*

Difference in frequency of extramedullary blood formation between subjects with *carcinomatous involvement* of all, and those with involvement of only some of the vertebrae

Differences

Between nodular and sinusoidal $p<0\ 001$
Between nodular and sinusoidal + nodular $p<0\ 001$
Between nodular and sinusoidal $0\ 02>p>0\ 01$
Between nodular and sinusoidal + nodular $0\ 01>p>0\ 001$
Between nodular and sinusoidal $p<0\ 001$
Between nodular and sinusoidal + nodular $0\ 01>p>0\ 001$
Between nodular and sinusoidal $0\ 01>p>0\ 001$
Between nodular and *sinusoidal* + *nodular* $p<0\ 001$
Between nodular and sinusoidal $p\approx0\ 01$

Nodular $p<0\ 001$

When all groups were pooled, 68 out of 99 (68 7 per cent) with carcinomatous involvement of the entire spine had extramedullary blood formation, the corresponding figures for the remaining subjects with skeletal metastases being 24 out of 93 (25 8 per cent) ($p<0\ 001$)

TABLE 4 *Incidence of Splenic Extramedullary Haemopoiesis and Bone Metastases with Different Patterns of Secondary Cancer in the Spleen (Gross & Marymont 1963)*

Pattern of splenic metastases	Number of cases	Number of cases with extramedullary blood formation	Number of cases with skeletal metastases
Nodular	61	13 (21 %)	34 (56 %)
Sinusoidal permeation	11	10 (91 %)	9 (82 %)
Diffuse	8	0	8 (100 %)
Total	80	23 (28 8%)	51 (63 0%)

sions, but of the two possibilities, *viz* that extramedullary haemopoiesis is a response to local factors (e g local hypoxia) depending on tumour cells in the sinusoids and that extramedullary haemopoiesis reduces the resistance of the spleen to the growth of tumour in the sinusoids, they thought the former more probable

The total frequency of extramedullary haemopoiesis and that of skeletal metastases in their series was largely the same as in that presented here Unfortunately, *Gross & Marymont* (1963) gave only the frequency of extramedullary haemopoiesis and that of skeletal metastasis, but no further details Analysis of the present series (Table 2) showed that while the total frequency was 38 0 per cent, the frequency among cases with skeletal metastases was 47 9 per cent (92/192) compared with 17 4 per cent (16/92) among cases without The difference was significant ($p<0\ 001$) The finding of so many cases of extramedullary haemopoiesis without coexisting skeletal metastases, however shows that also other factors are of significance

Two explanations may be offered for the relationship if any between splenic metastases and haemopoiesis in the spleen

I Splenic metastases stimulate haemopoiesis in the spleen *mainly in patients with skeletal metastases*

II Extramedullary blood formation facilitates tumour growth in the spleen *mainly in patients with skeletal metastases*

In both instances, then the occurrence of skeletal metastases is important and it is therefore desirable to know the extent of medullary destruction

It is clear from column I in Table 3 that extramedullary haemopoiesis is more common in cases with sinusoidal metastases (65 9 per cent) than in those with nodular metastases (28 3 per cent) This finding agrees with that by *Gross & Marymont* (1963) The difference was significant ($p<0\ 001$) A corresponding comparison between cases with skeletal metastases (column III) still showed a difference (72 2 per cent and 36 9 per cent, respectively), and the difference was still significant ($p<0\ 001$) Column IV shows, however, that involvement of the entire spine was more common in the cases with sinusoidal metastases than in cases with nodular metastases (66 7 per cent and 40 0 per cent, respectively) ($0\ 01>p>0\ 001$) and that among those cases with involvement of all the vertebral bodies (column V) the frequency of extramedullary haemopoiesis did not vary significantly with the type of metastases

It is clear from columns I–V that the group comprising both sinusoidal and nodular metastases was largely comparable to those comprising only sinusoidal metastases The difference from those comprising only nodular metastases was generally somewhat larger (than the difference between nodular sinusoidal), as reflected by the somewhat higher levels of significance of the differences found by comparison The higher frequency of skeletal metastases as well as of extramedullary haemopoiesis within the group compris-

33*

ing both sinusoidal and nodular metastases may be due to these cases being more advanced

Columns VI and VII give the cases without involvement of the entire spine and without skeletal metastases respectively. As regards the former, extramedullary haemopoiesis was demonstrated in 25.8 per cent (24/93), in the latter in 17.4 per cent (16/92). The difference was not significant and it therefore appears as if destruction of the bone marrow must be more extensive before it can have any influence on the extramedullary haemopoiesis in the spleen.

Since columns VI and VII do not differ from one another, they were pooled to form larger groups in the assessment of differences, if any, between the different types of metastases. In cases with nodular metastases extramedullary haemopoiesis was found in 16.9 per cent (27/160). For the same reason also the groups comprising sinusoidal metastases and those comprising sinusoidal + nodular metastases were pooled, upon which it was found that 52 per cent (13/25) had extramedullary haemopoiesis. The differences between the cases with only nodular metastases on one hand and those with sinusoidal or sinusoidal + nodular metastases on the other, was significant ($p<0.001$).

This means that among cases without skeletal metastases or with involvement of only some of the vertebral bodies the frequency of myeloid metaplasia varied with the metastatic pattern, a finding in agreement with that reported by *Gross & Marymont* (1963). In this respect the results differ from those found in an earlier investigation (*Berge* 1967) in which no statistically significant differences could be demonstrated in the frequency of extramedullary haemopoiesis with the type of metastases.

But the result should be judged with caution. Microscopic examination of the spine was not routine and it cannot be excluded that involvement of the spine has been missed or underestimated, especially in cases with diffuse leucotic metastasis. Such leucotic metastasis was often missed at gross examination of the spleen but was detected at routine histological examination. Such an erroneous assessment would mean that the destruction of the bone marrow in these patients was more advanced than supposed, which would explain the observed difference in extramedullary haemopoiesis. Moreover, the group is so small that a shift of even a few cases would be sufficient to eliminate the significance of the difference found.

In cases of carcinoma *Hennekeuser & Fischer* (1967) and *Fischer et al.* (1969) found extramedullary haemopoiesis in the spleen in 28 per cent of the cases with vertebral metastases, but in only 4 per cent of those without such metastases. They could not demonstrate any direct correlation between the extent of destruction of the bone marrow and extramedullary blood formation. Also in their series skeletal metastases appeared to be important. That they could not demonstrate any direct relation with the degree of skeletal destruction may be due to the smallness of their series. They thus had only 14 cases with skeletal metastases as well as extramedullary blood formation in the spleen.

According to *Wuketich* (1961) extramedullary haemopoiesis predisposes to diffuse malignant growth in the spleen, but the effect varies with the type of primary tumour. The predisposition is most pronounced in patients with carcinoma of the breast, bronchial carcinoma or melanoma, but least in cases of prostatic carcinoma.

Berge (1967) not only corroborated these relationships, but also showed that the metastatic pattern varied to a certain extent with the degree of differentiation of the tumour. Poorly differentiated tumours often set up secondaries in the spleen as well as in vertebral bodies and in many of these cases the entire vertebral column was involved. Poorly differentiated tumours occurred more often in the former group than in the group with prostatic carcinoma and this may explain the difference found by *Wuketich* (1961).

CONCLUSION

In carcinomas that have metastasized to the spleen, haemopoiesis in the spleen is more common in the presence than in the absence of coexisting skeletal metastases This is due mainly to the high frequency of extramedullary haemopoiesis in cases with extensive destruction of the bone marrow Other cases with skeletal metastases do not differ significantly from those without skeletal metastases This suggests that extramedullary haemopoiesis is a compensatory phenomenon, secondary to destruction of the bone marrow If the destruction of the bone marrow is extensive, extramedullary haemopoiesis does not vary with the type of metastasis

As regards the cases without skeletal metastases or with involvement of only a few of the vertebral bodies, the frequency of extramedullary haemopoiesis is higher among cases with sinusoidal metastases It was, however, not possible to draw any definite conclusions regarding the possible significance of the pattern of metastases in this group, in the first place because the group was small and secondly because it was not possible to assess the extent of involvement of the vertebral column with certainty

Extensive destruction of the bone marrow seems to be necessary before skeletal metastases can stimulate extramedullary blood formation But also other factors are important

Valuable assistance was given by *Arne Sundstrom* at the Computer Central University of Lund

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Acta path microbiol scand Sect A, 82, 514 518, 1974

MULTINODULAR STENOSING CARDIOVASCULAR AMYLOIDOSIS WITH INVOLVEMENT OF THE CONDUCTIVE TISSUE

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A case of multinodular stenosing cardiovascular amyloidosis in an elderly patient with progressive cardiac insufficiency is presented At autopsy, amyloid deposits were seen predominantly in the media of the intramyocardial arteries and in the arteries of the conductive system, but also in the media in arteries in the lungs, spleen, liver and kidney It is emphasized that the differential diagnostic is of importance in cases of this special form of intramyocardial vascular disease in elderly patients with an atypical cardiac history

In a review of amyloidosis which included a classification of cardiovascular amyloidosis (*Schwartz* 1970), a rare type was described and called multinodular stenosing cardiovascular amyloidosis (MSCA) The most serious complications of cardiovascular amyloidosis are cardiac insufficiency and arrythmia (*Jones & Frazier* 1950)

The purpose of the present paper is to describe the macro- and microscopic cardiac findings in the conduction system of the heart in a patient with MSCA in whom the clinical course was similar to that of a slowly progressive ischaemic heart lesion

CASE REPORT

An 80 year old woman who at the age of 54 developed malaise insomnia and precordial stabbing sensations The electrocardiogram at rest was normal The patient s physician noted arterial hypertension and started antihypertensive therapy In January of 1968, at the age of 75, the patient declared that she had had precordial pains of increasing intensity and worsening dyspnoea on effort for the last 20 years During the preceding two months there had also been a sensation of numbness in the fingers and pains in the upper extremities There were no hypertensive retinal changes and the electrocardiogram at rest was normal X ray of the chest revealed a broad cardiac silhouette 16 5/29 cm At the age of 76 the patient was hospitalized because of paraesthesia, malaise and pain in both upper extremities Symptoms were most pronounced in the left arm In spite of intensive investigation no specific explanation of these symptoms was found At the age of 77, hysterectomy and oophorectomy were performed because of endometrial adenocarcinoma The patient s complaints with regard to the upper arms and the cardiovascular system were unchanged Post-operative electrocardiography revealed Cohn effect, but now inverted terminal complexes were present in extremity leads as well as in precordial leads At the age of 80, the patient was hospitalized because of severe cardiac failure Electrocardiography revealed evidence of ischaemia with di phasic terminal complexes in the standard leads and ST depressions in the precordial leads together with atrial extrasystoles There was only slight response to treatment, the patient s condition deteriorated and she died of cardiac failure

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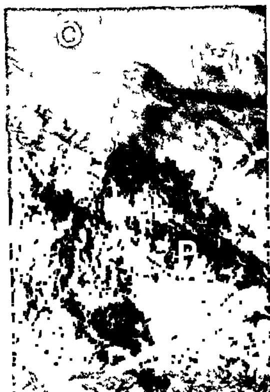


Fig 1 Left atrium with normal endocardium, uppermost (0) and wrinkled bark like endocardium near the mitral valve (P) Positive iodine-sulphuric acid test, × 2 5

Pathological Anatomy

At autopsy severe ulcerating aortic sclerosis was found, together with acute and chronic pulmonary stasis chronic stasis in the liver and spleen as well as atherosclerotic and atheriolosclerotic changes in the kidneys

The heart was hypertrophic and slightly dilated weighing 440 grams The thickness of the right ventricle was 5 mm, that of the left ventricle was 18 mm The origin of the coronary arteries was normal and diffuse atherosclerosis of the elastic type and with local calcification was found but there was no evidence of stenosis or occlusions Calcification of the aortic valve leaflets was seen but no stenosis The left atrium was moderately dilated and the endocardium thickened dull and grooved The endocardial changes gave a strong positive reaction on staining with iodine sulphuric acid (Fig 1) Similar changes were seen in the right atrium In the left ventricle, and to a lesser degree the right ventricle the myocardium contained numerous grey noduli measuring 2 3 mm These noduli also stained strongly with iodine sulphuric acid (Fig 2) The papillary musculature was characterized by atrophy and fibrosis, but the chordae tendineae were thin and not fused

The conduction system of the heart was studied according to the system of *Davies* (1971) Sections from the liver, kidney, spleen, lungs and myocardium including the conduction system were stained with haematoxylin eosin, van Gieson Hansen s connective tissue stain, methyl violette, sirius red (*Sweat & Puchtler* 1966) basic Congo red (*Puchtler & Sweat* 1962) Slides stained with basic Congo red were investigated under polarized light in order to detect green dichroism In addition fluorescence microscopy was carried out with transmitted light using fluorochrome thioflavin T (*Vassar & Culling* 1959) Sections from the myocardium and the conducting system were also stained with haematoxylin basic fuchsin picric acid (HBFP, *Lie et al* 1971)

Microscopically, a slight variation in the width of muscular fibres and the nuclear size was found in the myocardium There was marked perivascular fibrosis with moderately pronounced myofibrosis Staining by which to detect ischaemia (HBFP) gave a positive reaction In numerous intramyocardial arteries deposits of an acellular homogeneous substance were noted, usually in the form of

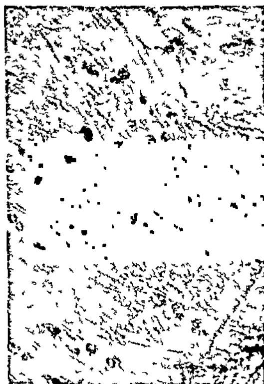


Fig 2 Anterior wall of left ventricle with focal areas of nodularity Positive iodine sulphuric acid test, × 2 5

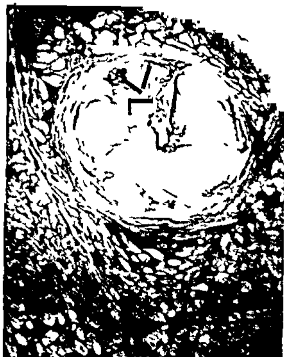


Fig 3 Intra myocardial artery with nodular amyloid deposits (L = remaining lumen) Thioflavin T, × 80

nodules in the media, but also in the adventitia and intima The lumina of several arteries were severely stenosed (Fig 3) A similar substance was also found in the veins, but to a lesser degree The subendocardium especially in the left atrium, revealed large deposits of an acellular homogeneous substance In the musculature of the atria and the ventricles smaller focal areas with an interstitially deposited substance were seen and in the conduction system deposits were seen in the artery to the sinus node (Fig 4), sparingly in the artery to the atrioventricular node and pronounced in the media of arteries supplying the bundle of His and, in addition, interstitially near the bifurcation of the bundle of His (Fig 5)

In the liver, spleen lungs and kidneys similar deposits were seen in arteries predominantly located in the media. The lumen of these arteries was preserved No deposits were seen in the parenchyma of these organs In all specially stained preparations, the acellular and homogeneous substance revealed histo chemical reactions consistent with amyloid

DISCUSSION

Schwartz (1970) classified cardiovascular amyloidosis as follows

1 multi nodular, stenosing amyloidosis of the cardiac and other extracerebral vessels
2 diffuse interstitial, fibrillary cardiac amyloidosis
3 multi focal massive interstitial amyloidosis *with degeneration of cardiac muscle*
4 diffuse massive interstitial cardiac amyloidosis

The amyloidosis demonstrated in the present case can be classified under type 1 In the present case there was no evidence of secondary amyloidosis, but multiple myeloma must be considered Hypoproteinaemia with hypo

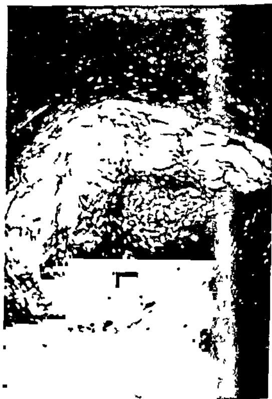


Fig 4 Artery to sinus node with crescent shaped amyloid deposits (l = lumen) *Thioflavin T* × 80



Fig 5 Left mean branch of conducting system indicated by markings Amyloid deposits are seen within and outside the conducting system and the vessels Thioflavin T, × 80

gammaglobulinaemia was found and a normal serum calcium, X ray of the thoracic spine showed normal conditions These findings do not suggest classical myeloma, but studies are insufficient since immuno-electrophoretic examinations of urine and serum were not undertaken At autopsy there was no evidence of myeloma and signs of osteolytic processes in the spinal collumn or the ribs were not found Microscopic examination of the bone marrow revealed an increased number of plasma cells, approximately 20 per cent, but no abnormal forms

The histological changes of nodular deposits in the intramyocardial arteries suggest primary amyloidosis Similar typical deposits of amyloid substance in dogs have previously been described (*Hjärre* & *Nordlund* 1942), and it has been maintained that a special form of atherosclerosis in dogs was involved (*Lindsay et al* 1952) Nodular vascular amyloid deposits in man have previously been described (*Brocher* 1932) *Schwartz and co workers* (1970) report on the basis of their studies that these changes are more common in elderly individuals than previously suspected and that this form of amyloid deposits has been neglected in the literature

Amyloid deposits in the artery of the sinus node and in the interstitial atrial tissue provide an adequate explanation of the atrial extrasystoles seen in the patient Diffuse amyloid deposits in the conductive system, like those previously described (*James* 1966), were not seen in this patient The marked amyloid deposits in the intra myocardial arteries with severe luminal stenosis probably resulted in a low perfusion syndrome with cardiac ischaemia followed by pump failure By special staining (HBFP), clear evidence of ischaemia was obtained, but no necrosis was noted

The patient s paraesthesia in the upper extremities was probably caused by amyloid deposits (*Jones* & *Frazier* 1950)

This study was supported by grants from the *medical research funds of the county hospitals in Ringkøbing, Ribe and Sønderjylland* and from *Maria de Lancy Pedersen* s foundation

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Acta path microbiol scand Sect A 82, 519 533, 1974

LOBULAR CARCINOMA *IN SITU*

A Long Term Follow-up in 52 Cases

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During a retrospective histological study based on examination of 3299 female breast tissue specimens with otherwise benign diseases, 52 cases of lobular carcinoma in situ were found Five cases presented previous invasive breast carcinoma, and one of these developed subsequently invasive carcinoma during the first year after the biopsy Out of 47 cases without previous invasive breast carcinoma, 10 developed subsequently 12 invasive carcinomas Eight were ipsilateral and were revealed within the 1st, 2nd, 3rd, 5th, 7th, 14th, 18th, and 22nd year, respectively Four were contralateral and were found within the 7th, 8th, 12th, and 24th year after the primary biopsy The average follow up period was 15 years Strict follow up of patients with lobular carcinoma in situ, carried out by a permanent team, is considered a realistic alternative to mastectomy

Very few long term studies of patients with lobular carcinoma in situ of the breast have been reported (*Hutter & Foote* 1969, *Haagensen et al* 1972) The commonly accepted treatment consists of simple or modified mastectomy (*Foote & Stewart* 1941, *Godwin* 1952, *Neumann* 1963, *Neumann* 1966, and *McDivitt et al* 1967), and the possibility of collecting new prospective series is therefore limited For this reason, investigations must necessarily be based on material from a period when the nature of the disease remained unrecognized, or when discovery of the lesion did not give rise to such radical treatment as mastectomy

The present study was prompted by the wish to compare a European series, with the longest possible period of observation, with the available American series which have greatly influenced the attitude towards this disease Furthermore, it was desired to draw the attention of both clinicians and pathologists to the special problems presented by this disease

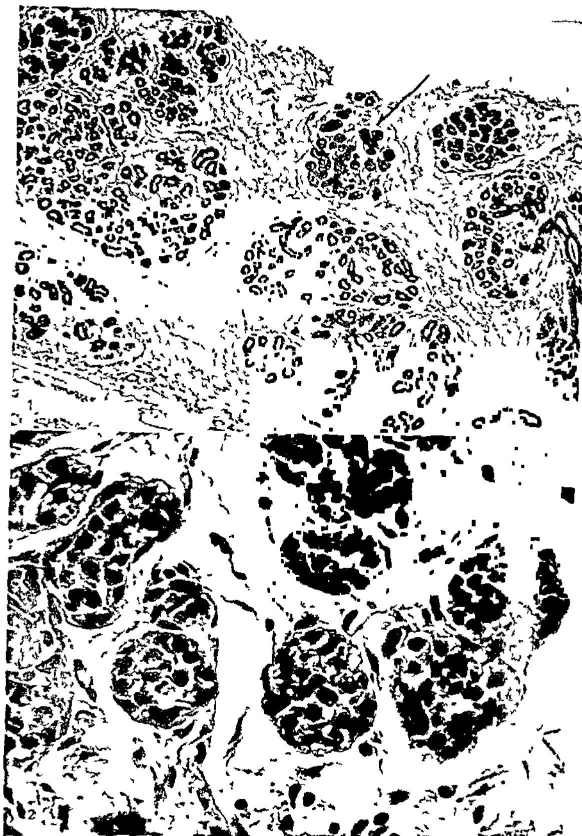
MATERIAL AND METHODS

The cases that comprise the data for this study were 52 females with lobular carcinoma in situ of the breast without concurrent invasive breast carcinoma The series originates from the Institute of Pathology, Bispebjerg Hospital, from 1 January 1942 to 30 September 1961 and was selected *retrospectively* by histological review of all breast tissue specimens classified as benign at the primary histological study During the period concerned the Institute did not use the term lobular carcinoma in situ, and only occasionally were the actual changes described A total of 5278 recently produced slides derived from 3299 breast tissue specimens were examined Only in 89 cases was it not possible to trace the original tissue blocks Hence, 1 6 tissue blocks per breast were examined This corresponds most likely to the original number since, during the period concerned, only 1–2 tissue sections per breast biopsy were usually studied

All cases in which one single lobule fulfilled the following criteria as to lobular carcinoma in situ, were included in the series obliterating epithelial

Received 25 ii 74 Accepted 6 iii 74

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proliferation in lobular ductules with or without involvement of the terminal lactiferous duct The proliferating cells were larger than the normal epithelial cells and without polarity The normal epithelial stratification was lost. The nuclei were relatively large, very uniform, rounded and not particular hyperchromatic, and the cytoplasm was pale, eosinophilic and often vacuolated The cellular cohesion was often poor (Figs 1 10) The first four illustrations show the minimum demand which had to be fulfilled before a case could be included in the study

From past clinical case records, all data concerning menstrual history, pregnancies, hormone therapy, present and previous diseases, symptoms from breasts and the physical examination of the breasts and treatment were obtained It was then checked whether the case had at any time been notified to the Danish Cancer Registry for malignant neoplasm and, in particular, for invasive breast carcinoma Furthermore, the extract, if any, from the Danish Cancer Registry, showed the side of the carcinoma, the time of diagnosis, and the place of treatment It was then possible, from more recent case records, to check the above data and at the same time to obtain histological specimens of the invasive carcinoma without having to contact the patients and give rise to unnecessary anxiety By means of the national registers it was possible to check up on all personal data and also to get copies of death certificates, if any If autopsy had been carried out, copies of the records were also available In cases where invasive breast carcinoma had been diagnosed and treated prior to the actual biopsy, and in cases where ipsilateral, contralateral, or both ipsilateral and contralateral invasive breast carcinoma had developed later, the histological specimens were type classified in accordance with the WHO Classification (1968) Predominance of a haphazard arrangement of individual cells and locations with single file arrangement of cells was required in order to establish the diagnosis of invasive lobular carcinoma. Often the individual cells or the one cell wide rows of cells was arranged in a concentric fashion around ducts following the natural course of the fibrous tissue (Figs 11 12)

Fig 1 Section of breast biopsy from a 39 year-old woman who developed ipsilateral invasive carcinoma 1½ years later The lesion was demonstrated only in the lobule indicated by the arrow This shows how unobstrusively lobular carcinoma in situ can appear in between normal lobules in survey magnification Haematoxylin-eosin 73×

Fig 2 Section of Fig 1 corresponding to the arrow shows the lobule with massive epithelial proliferation in the lobular ductules with destroyed stratification and enlarged, rounded, rather uniform nuclei Such lesions were classified as lobular carcinoma in situ and fulfill the minimum requirements Haematoxylin-eosin 730×

A further review of the histological findings in lobular carcinoma in situ, the relationship with other lesions of the breast and any development of invasive carcinoma will be dealt with in a forthcoming paper

RESULTS

During the retrospective study of breast tissue from 3299 patients, a total of 52 female patients were found who had lobular carcinoma in situ This corresponds to an frequency of 1 5 per cent of lobular carcinoma in situ in patients with otherwise benign breast disease

The average age of the patients at the primary operation was 46 years (range 20–76 years) As will be seen from Table 1, 40 (77 per cent) were in the 4th or 5th decade

The most essential clinical data are shown in Table 2 Eighteen patients, 10 of whom were single, had not completed a pregnancy, but 3 had had a miscarriage

In 28 cases the patient's own finding of a lump in the breast led to examination and surgery In 17 cases, 5 of whom had hypertrophic breasts, the patient complained of pain and/or tenderness, and in only one case was there bloody nipple discharge In 6 cases a tumour was discovered at routine examination or check-up The symptoms had been present for less than one week in 3, between 2 and 4 weeks in 10, between 5 weeks and 3 months in 8, between 4 and 6 months in 3, between 7 and 12 months in 8, and for longer periods in 4 cases In 5 cases the data were too inaccurate, in 5 cases the symptoms of hypertrophy had been present for several years, and 6 cases were discovered at check up Eighteen patients had previously undergone surgical excision for benign breast disease and 5 had had simple mastectomy and radiotherapy for invasive carcinoma

The site of the breast tissue and/or tumour removed appears from Table 3 Hence, in 7 cases the site was definitely within the limits of the lower quadrants, whereas in 27 cases the lesion was situated within the limits of

3

4

TABLE 1 *Age Distribution of 52 Females with Lobular Carcinoma In Situ*

20 29 years	30 39 years	40–49 years	50–59 years	60 69 years	70–79 years	Total
3	5	29	11	1	3	52

TABLE 2 *Clinical Information on 52 Females with Lobular Carcinoma in Situ*

	Number of cases
Previous operation for benign breast disease	18
Females with full term pregnancy	20
Total number with full term pregnancy	40
Females with miscarriage	10
Menstrual irregularities	11
Hormone therapy	4
Incomplete history	14

TABLE 3 *Localization of Biopsy and/or Tumour in 52 Females with Lobular Carcinoma in Situ*

	Number of cases
Mammary gland	
right	22
left	29
unknown	1
Quadrants	
upper outer	17
upper inner	6
lower outer	3
lower inner	0
between uppers	4
between lowers	4
between outers	8
between inners	1
subareolar	2
unknown	7

the upper quadrants The 5 cases in which plastic surgery was carried out are not included in the 27 cases

In 36 cases the tumour was localized, whereas in 7 cases more diffuse lesions were seen In 4 cases the data were too inaccurate, and 5 patients with breast hypertrophy had no localized tumour The physical signs were those of benign breast disease and none of the characteristics of obvious malignant disease was noted

The gross description of the breast tissue removed was in all cases rather brief, mostly reading as follows whitish grey tough tissue with or without cysts None of the descriptions denoted invasive carcinoma, and in no description was the appearance of large lobules mentioned (*Foote* & *Stewart* 1941) In 22 patients macroscopical cysts were found

The nature of the surgical procedure appears from Table 4 During the plastic surgery, attempts were made to remove most of the glandular tissue in the upper quadrants, while the nipple with the subareolar tissue and most of the glandular tissue in the lower quadrants were preserved (personal communication, *Bandier* 1974) Hence, the surgical procedure accords with upper quadrectomy

In 5 cases invasive carcinoma had been diagnosed 4 and 2 years earlier, and in 3 cases less than one year earlier One of these patients subsequently developed invasive carcinoma in the remaining breast

In the 47 cases who had not previously been treated for invasive breast carcinoma, as

Fig 3 Section of breast biopsy from a 42-year-old woman who had not developed invasive carcinoma 17 years after the primary biopsy The five proximal lobules, the outer left being indicated by an arrow, are not totally enlarged, but a few of the lobular ductules are Haematoxylin-eosin 73×

Fig 4 Section of Fig 3 corresponding to the arrow shows massive epithelial proliferation in the lobular ductules without stratification and cellular polarity The enlarged nuclei are round and rather uniform and the cellular cohesion is poor These lesions too are classified as lobular carcinoma in situ and fulfill the minimum requirements Haematoxylin-eosin 730×

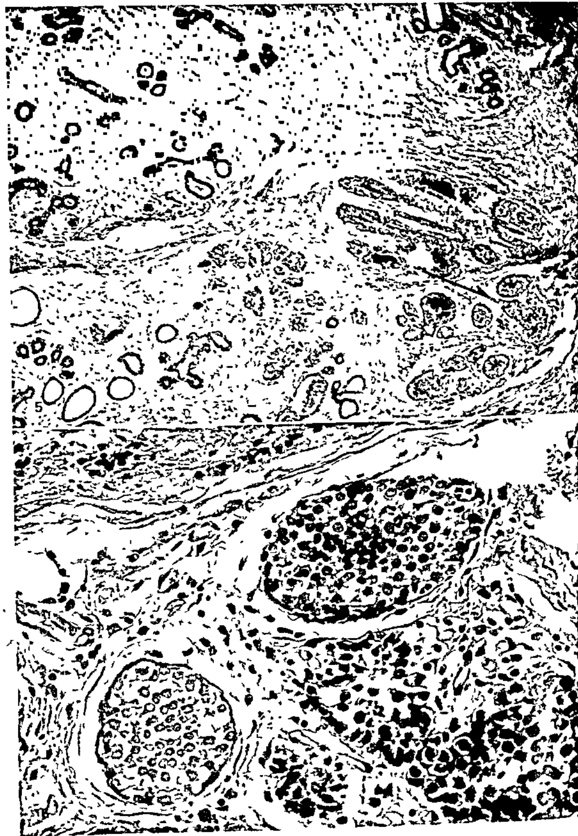

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TABLE 4 *Surgical Procedure, Number of Breasts Available in Follow up and Number of Cases of Subsequently Developed Invasive Breast Carcinoma in 52 Females with Lobular Carcinoma in Situ*

	Number of cases	Breasts available for follow up		Subsequently developed invasive carcinoma	
		ipsilateral	contralateral	ipsilateral	contralateral
Extirpation, resection	41*	41	38	9	4
Simple mastectomy	6†	0	4	0	0
Plastic operation (bilateral)	4	4	4	0	0
Plastic operation (unilateral)	1	1	1	0	0
Total	52	46	47	9	4

Three* and two† females, respectively, had undergone previous contralateral mastectomy for invasive carcinoma

appears from Table 4, a total of 12 cases of invasive carcinoma were found in 10 patients Eight of these were ipsilateral and were found during the 1st, 2nd, 3rd, 5th, 7th, 14th, 18th, and 22nd year after the primary biopsy Four were contralateral and were diagnosed during the 7th 8th, 12th, and 24th year after the primary biopsy Consequently, 2 patients developed bilateral invasive carcinoma, in one case 4 and 7 years after the primary biopsy, and in the other case, 16 and 22 years later None of the five cases in which plastic surgery was carried out and where the period of observation ranged from 13 to 28 years on an average 18 years developed invasive breast carcinoma

Hence, amongst the 52 patients there were 5 previously developed and 13 subsequently developed invasive breast carcinomas in 15 patients The type of carcinoma in the 18 cases appears from Table 5 In 9 cases it was invasive lobular carcinoma, in one case both invasive lobular and ductogen carcinoma, and in 6 cases ductogen carcinoma In the remaining 2 cases, the specimens were too small and too badly damaged to allow of a definite classification, although lobular carcinoma in situ was revealed in one case

The follow-up period, counted from the date of the primary biopsy and to the end of 1971, had been between 2 and 28 years, or on an average 15 years for the 52 patients Ten had died during the 1st, 2nd, 3rd, 4th, 6th, 7th, 10th, 13th, and 24th year after the primary biopsy One died from congestive heart disease, two from pneumonia, one from rectal carcinoma with metastases, one from previous invasive contralateral carcinoma and one from bilateral invasive breast carcinoma 6 years after the discovery of the last carcinoma The last 4 patients died from subsequently developed invasive breast carcinoma with metastases 1, 9, 12, and 23 years after the primary biopsy had shown lobular carcinoma in situ

Apart from breast carcinoma, another four malignant tumours developed in the 52 patients 2 in the uterus, one in the rectum, and one in the stomach

Fig 5 Section of breast biopsy from a 42 year-old woman who developed ipsilateral invasive carcinoma 6 years later Similar lesions were found in other areas The enlarged lobules lie between apparently normal lobules and one of the enlarged lobules is cut longitudinally Haematoxylin-eosin 73 ×

Fig 6 Section of Fig 5 corresponding to the arrow Transversely cut lobular ductules with lesions characteristic of lobular carcinoma in situ The cellular cohesion is greatly reduced Haematoxylin eosin 456

DISCUSSION

From the early papers concerning lobular carcinoma in situ (*Foote & Stewart* 1941 *Godwin* 1952, *Barnes* 1959), it appears that

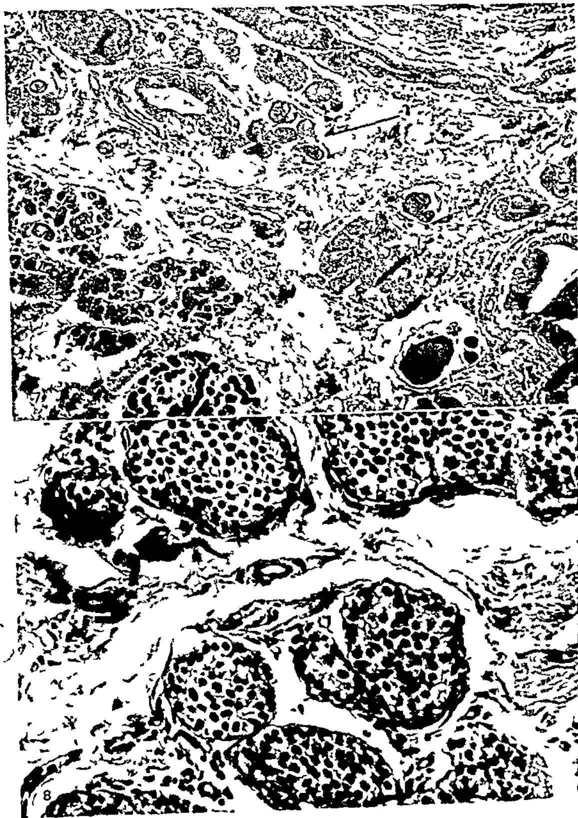

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TABLE 5 *Type of Invasive Carcinoma in 18 Developed Cases, 13 Subsequent and 5 Previous*

No	Subsequent invasive carcinoma	Previous invasive carcinoma	Invasive lobular carcinoma	Other types of invasive carcinoma	Lobular carcinoma in situ concurrent with invasive carcinoma
3	+		+	+	+
8	+		+		+
8	+		+		
11	+			+	
13	+			+	+
23		+	?	?	+
24	+		+		+
31	+		?	?	
31	+			+	
32		+	+		+
32	+		+		+
34	+			+	+
35		+		+	+
36	+		+		+
37		+	+		+
39	+		+		+
40	+			+	
42		+	+		+

the lesion was uncommon, although the frequency was not stated Among 7500 breast biopsies, *Neumann* (1963) found 32 cases in 26 females, but the ratio of benign and malignant breast biopsy is not reported In the more comprehensive long term follow-up series, which was described both by *McDivitt et al* (1967) and by *Hutter & Foote* (1969), or in the cases presented by *Haagensen et al* (1972), the frequency of lobular carcinoma in situ was not given *Haagensen et al* (1972) mentioned that the frequency was low and that the frequency of 1 5 per cent reported by *Lombird & Shelley* (1969), in biopsies from patients with otherwise benign breast disease, was much higher than that found in their laboratory *Lombird & Shelley* (1969) reported the frequency found by them as being caused by the fact that they withdrew up to 8 tissue sections from a adequate volume of breast tissue (15 20 cc) *Giordano & Klopp* (1973) found a frequency as high as 2 5 per cent They revealed 124 cases of lobular carcinoma in situ in 8000 biopsies During the period up to and including 1961 they found a frequency of 1 per cent From 1964 onwards, however, the frequency found remained almost stationary at 2 5 per cent, due to the fact that from that year they examined an average initial number of 10 tissue blocks from each breast biopsy The frequency of 1 5 per cent found during the present study must be considered on the basis of the fact that an average of only 1 6 blocks of tissue were examined per breast biopsy Hence, the actual frequency in Copenhagen is most likely higher than that found during the present study

As was the case in the studies carried out by *Hutter & Foote* (1969) and by *Haagen-*

Fig 7 Section of breast biopsy from a 52 year old woman who had not developed invasive carcinoma 15 years after the primary biopsy In this section all lobules present more or less pronounced changes and are enlarged Similar epithelial changes are seen in a few terminal lactiferous ducts Haematoxylin eosin 73 ×

Fig 8 Section of Fig 7 corresponding to the arrow shows the typical and almost monotonous changes which characterize lobular carcinoma in situ Haematoxylin-eosin 456 ×


10

sen et al (1972), the diagnosis of lobular carcinoma in situ was established by histological examination, no clinical signs or physical findings indicating this disease in particular or any other malignant breast disease. Contrary to the other studies, the diagnosis in the present study was made by retrospective review. Hence, the clinical data are almost exclusively derived from the primary case records, and no attempts were made to elaborate such data by questioning the 42 patients who are still alive. Therefore only limited value can be attached to the common case histories. However, it is remarkable that out of the 20 patients with full term pregnancies 9 developed invasive carcinoma whereas only one out of 18 without full term pregnancies developed this lesion. On the whole, these figures are completely inconsistent with the theory that pregnancy, and in particular an early first full term pregnancy, should protect against later development of invasive carcinoma (*Mahon et al* 1973). It is also suggestive that 18 patients (39 per cent) had previously undergone surgery because of benign breast disease (most often classified as fibroadenomatosis), this frequency being considerably higher than the 5 per cent found by *Mahon et al* (1973) in invasive breast carcinoma in a recent population based study. This condition, and also the fact that both diseases are most often found during the premenopausal period, may indicate a common genesis of fibroadenomatosis and lobular carcinoma in situ. A re-evaluation of these 18 biopsies will be included in a forthcoming paper.

The remaining well known essential risk factors for human breast cancer cannot be assessed by means of the rather limited series available.

Antonius & Jones (1963) found the upper outer quadrant to be the site of lesion in 19 out of 31 patients. This is in accord with the fact that 60 per cent of all invasive carcinomas are situated in this area (Urban 1967). *Lombird & Shelley* (1969) reported the distribution of the foci of lobular carcinoma in situ in 9 breasts studied in 2.5 cm step blocks, the tissue in 8 cases being completely embedded. In 50 per cent of the cases they found the lesion to be concentrated in the upper quadrants, within 5 cm from the nipple; in cases where the foci were situated more peripherally, they were often concentrated corresponding to single lobes. *Haagensen et al* (1972) did not find the distribution of lobular carcinoma in situ to be so simple. Also in this study, lobular carcinoma in situ was found to be much more frequent in the upper quadrants (27 against 7), and to occur most often in the outer quadrant. Hence, in blind biopsy excision it must be rational to choose the upper (outer) quadrants and, as mentioned by *Lombird & Shelley* (1969), to remove the breast tissue through a circumareolar incision in a 1 to 2 cm tissue border between 9 and 3 o'clock about 3–4 cm from the nipple. If all the tissue is embedded, the chance of finding lobular carcinoma in situ if this lesion is present at all in the breast examined should be about 90 per cent.

The most essential problem as to how often patients with lobular carcinoma in situ will

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Fig. 9. Section of breast biopsy from a 42 year-old woman who developed invasive carcinoma in the contralateral breast 6 years later. She died 3 years later with metastases. At autopsy the breast from which the primary biopsy specimen was obtained was without tumours. In this section the lesions are diffuse without intermediary normal lobules. Alcian blue (*Eskelund* 1957). 73 ×.

Fig. 10. Section of Fig. 9 corresponding to the arrow. Typical lobular carcinoma in situ with almost monotonous appearance. Alcian blue (*Eskelund* 1957). 456 ×.

Fig. 11. Section of invasive lobular carcinoma from the case presented in Figs. 9 and 10. Note the diffuse cellular infiltration which tends to concentrate around the original glandular structures. Haematoxylin eosin. 73 ×.

Fig. 12. Section of Fig. 11 showing small cells with dark nuclei and sparse inconspicuous cytoplasm. The epithelial cells lie singly or in Indian file following the structure of the connective tissue. Note that the cells are concentrically arranged around the original glandular structures. Haematoxylin-eosin. 456 ×.

TABLE 6 *Comparison of Follow up in Three Series of Females with Lobular Carcinoma in Situ without Previous or Concurrent Invasive Breast Carcinoma*

	Hutter & Foote (1969)	Haagensen et al (1972)	Authors	Total
Number of cases	46	47	43*	136
Length of follow up	4 27 years	4 24 years	2 28 years	2 28 years
Mean length of follow up	?	9 years	15 years	?
Number of ipsilateral breasts available for follow up	40	22	38	100
Subsequent invasive carcinoma in ipsilateral breasts	10	5	8	23 (23 0 %)
Number of contralateral breasts available for follow up	46	47	43	136
Subsequent invasive carcinoma in contralateral breasts	4	5	4	13 (9 5 %)
Total number of breasts available for follow up	86	69	81	236
Total number of subsequent invasive carcinoma of breast	14 (16 3 %)	10 (14 5 %)	12 (14 7 %)	36 (15 2 %)
Number of females with subsequent invasive carcinoma	11	9	10	30 (22 0 %)
Number of females who died with metastases from invasive carcinoma of the breast	2	0	4	6 (4 4 %)

* 5 females with previous invasive breast carcinoma and 4 with bilateral plastic operations are excluded in an attempt to obtain comparable material

develop invasive carcinoma has been examined in very few studies *Benfield et al* (1965) reported on 13 patients with lobular carcinoma in situ, 8 of whom underwent ipsilateral and another 3 also contralateral mastectomy The period of observation was between 6 months and 30 years on an average 6½ years and in none of the cases did invasive carcinoma develop However, according to *Giordano & Klopp* (1973) (personal communication from *Benfield* to *Giordano & Klopp* 1973) 3 patients have subsequently developed invasive carcinoma and further more one of them has *metastases*. *Farrow* (1970 showed that out of 470 patients with non infiltrating carcinoma from the period 1949 1967 236 cases were lobular carcinoma in situ and 34 a combination of this lesion and cancer in situ which apparently arose in the terminal ducts Some of the patients had previously had invasive carcinoma of the opposite breast Forty one patients had been treated exclusively with local excision and only 2 are known to have developed ipsilateral invasive carcinoma subsequently, both within a 5 year period

Giordano & Klopp (1973) mentioned that 19 of their patients had refused mastectomy and therefore had been given very frequent check ups over more than 3 years Two developed ipsilateral invasive carcinoma subsequently after 3 and 6 years respectively

In the present study the follow up was carried out by help of the Danish Cancer Registry to which practically all cases of malignant diseases occurring in Denmark are notified The follow up can be considered to be complete up to the end of 1971 at which time all data on the cases notified were available

In Table 6 the most important results of the three comprehensive sets of material are presented collectively *Five cases of previous* invasive breast carcinoma and 4 cases with bilateral plastic operations included in the present study were excluded in order to ob-

tain a comparable series The frequency of subsequent development both of ipsilateral and contralateral invasive carcinoma shows great similarity It is remarkable, however, that the average period of observation in the present material is 15 years, whereas *Haagensen et al* (1972) had a period of observation of 9 years, and yet this difference does not give rise to any higher frequency of invasive carcinoma in the present material This may be incidental, but it is hardly caused by differences in the histological selection procedure (see Figs 1–10) It should also be borne in mind that the patients included in this material, as far as is known, have not been followed consistently each third month and that some of the 42 surviving patients might be on the verge of developing recognizable carcinoma

In the present series two invasive carcinomas developed ipsilaterally within the first year after demonstration of lobular carcinoma in situ From the knowledge of constant doubling times for the tumour (*Collin et al* 1956, *Schwartz* 1961), the invasive carcinomas existed most likely already at the time when biopsy was taken Because neither of the two cases was clinically recognizable, and could not even be demonstrated at surgical exploration, they are included in the present series

A total of 3 patients developed bilateral invasive carcinoma, and in all of them the carcinoma was situated in the glandular tissue itself In none of the cases was sulcus intermammalis affected, and when the last invasive carcinoma was diagnosed only one patient had axillary nodular metastases and also bone metastases In one case the first carcinoma could not be classified, while the second invasive breast carcinoma was typically ductogen with maintained intraductal carcinoma lesions All four invasive carcinomas in the other two cases were of the lobular type with definite remnants of lobular carcinoma in situ in three cases and doubtful remnants in the fourth case, although only one specimen was available Hence in none of the cases was the secondary carcinoma considered to be metastases from the earlier invasive breast carcinoma, they were all taken to be primary carcinomas (*Robbins & Berg* 1964, *Haagensen* 1971)

In one patient with asynchronous bilateral invasive carcinoma, autopsy revealed also diffuse anaplastic stomach carcinoma (linitis plastica) While it is common knowledge that primary uterus carcinoma is a frequent finding in patients with breast carcinoma (*Haagensen* 1971), the relationship between primary breast carcinoma and stomach carcinoma is considered to be extremely rare in the western part of the world, but quite common in Japan (*Yoshida* 1973) The possibility of metastases (secondary linitis plastica) must be considered (*Fischermann* 1957)

Invasive lobular carcinoma amounts generally to about 5 per cent of all breast carcinomas (*Newman* 1966, *Ashikari et al* 1973) In cases where lobular carcinoma in situ has been found earlier, as in the present study and in the other two series (*Hutter & Foote* 1969, *Haagensen et al* 1972), the frequency of the lobular type of carcinoma is about 50 per cent

The most commonly recommended treatment for lobular carcinoma in situ is simple mastectomy of modified mastectomy (*Foote & Stewart* 1941, *Stewart* 1950, *Godwin* 1952, *Barnes* 1959, *Newman* 1963, *Benfield et al* 1965, and *Newman* 1966), and, furthermore, contralateral biopsy from the upper outer quadrant (*Newman* 1963, *Benfield et al* 1965, *McDivitt et al* 1968, and *Lewison & Finney* 1968) However, *Haagensen* (1962) was of the opinion that, on the basis of the experience obtained at that time, it was only possible to make a guess as to the correct therapy The reason for recommending ipsilateral mastectomy was that it was known that the disease in many cases was multicentric Thus, *Benfield et al* (1965) proved that the mastectomy cases reported in the literature at that time, a total of 44 cases, had showed residual lobular carcinoma in situ in 39 of the cases This has subsequently been proved by *Lewison & Finney* (1968) and by *Warren* (1969), who concluded that the frequency

of multicentricity ranged at about 70 per cent The question as to whether the lesion would be bilateral, both synchronous and asynchronous, was first advanced by *Barnes* (1959) and later by *Newman* (1963) who found that 6 out of 26 cases were bilateral, and by *Benfield et al* (1965) who found that 2 of their 5 cases were also bilateral *Warren* (1969) concluded that not less than 15 per cent were bilateral *Urban* (1967), however, found a considerably higher frequency of 35 per cent, and *McDivitt et al* (1968) considered the frequency to be about 30 per cent The problem of bilateral appearance of lobular carcinoma in situ in the present material will be dealt with in a fortcoming paper The allround consequence of the incidence of both multicentricity and bilateral appearance of lobular carcinoma in situ caused *Benfield et al* (1972) to recommend even primary bilateral mastectomy *Haagensen et al* (1972) took a more moderate and expectant attitude and the present study seems to support their ideas, in particular when the much longer follow up period in my series is borne in mind As far as is known, the patients included in the present series have not been checked consistently each third month and the fact four of them died because of subsequently developed invasive breast carcinoma is more significant as to the natural course of the disease than the results which would have been obtained by a more careful follow up of the patients Indeed, none of the patients included in the series examined by Haagensen et al (1972), died from invasive breast carcinoma

Also others (*Kaufmann et al* 1971, *Herrmann* 1972) now seem to take up a more conservative attitude to lobular carcinoma in situ

A therapeutic alternative to ipsilateral mastectomy or even to bilateral mastectomy would be a life long follow up of the patients by a permanent team comprising surgeon, radiologist and pathologist

The knowledge of the site of the lobular carcinoma in situ and to a lesser extent the experience obtained from our five cases in whom surgery corresponding to upper quadrectomy was performed, might possibly speak in favour of upper quadrectomy and life long follow-up if the lesion is found in this region

CONCLUSION

1 Lobular carcinoma in situ represents at least 1 5 per cent of the otherwise benign breast biopsies from Copenhagen

2 No clinical symptoms or objective signs indicate lobular carcinoma in situ The lesion is found incidentally by histological examination

3 Lobular carcinoma in situ is demonstrated most frequently in the upper quadrants, and blind biopsy excision should be made through a circumareolar incision in this area

4 When the long-term follow up period was between 2 and 28 years, on an average 15 years the frequency of subsequent invasive carcinoma per breast was about 14 per cent 19 5 per cent ipsilateral and 8 5 per cent contralateral respectively

5 The ipsilateral invasive carcinomas developed within the 1st, 1st, 2nd, 3rd 5th, 7th 14th, 18th, and 22nd year, and the contralateral cases within the 7th 8th 12th and 24th year after primary biopsy of the lobular carcinoma in situ

6 Strict follow up of the patients with lobular carcinoma in situ by a permanent team is considered a realistic alternative mastectomy

The author is grateful to Dr *Charles Johansen* M D, Chief Pathologist for kindly permitting the use of breast tissue specimens from the Institute of Pathology, Bispebjerg Hospital, and to Dr *Johannes Clemmensen*, M D, Chief of the Danish Cancer Registry for valuable assistance Furthermore my thanks are due to Dr *William Kiær* M D Chief Pathologist and Dr *Knud Erik Sjølin* M D Chief Pathologist for their helpful advice and encouragement

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Acta path microbiol scand Sect A, 82, 534–538, 1974

LONG TERM TOXICITY STUDY WITH ALCOHOL AND 4-METHYLPYRAZOLE IN RAT

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4 Methylpyrazole, in a dose producing a partial inhibition of the alcohol dehydrogenase activity, was given together with alcohol by peroral administration during 12 weeks in growing rat The effect of similar doses of alcohol and 4 methylpyrozole alone were also investigated Light microscopy of liver, kidney, heart and pancreas revealed no changes related to the administration of 4 methylpyrazole, furthermore, serum transaminases were normal The administration of alcohol caused decreased weight gain of the rats, supplementation with 4 methylpyrazole made this decrease still more pronounced Fatty change in the livers was conspicuous in rats receiving alcohol alone, and less pronounced after supplementation with 4 methylpyrazole There occurred a few cases with a moderate increase in serum creatinin both in rats receiving alcohol and 4 methylpyrazole, and in those receiving alcohol alone There were no light microscopic changes in the kidneys of these rats Although no definite changes related to the treatment demonstrated, it is concluded that further investigations are needed before 4 methylpyrazole can be used in adequate repeated doses in clinical investigations in man

Theorell et al (6, 10, 11) found pyrazole and some of its substituted derivatives to be potent inhibitors of the liver alcohol dehydrogenase (LADH) This opened new possibilities for studies on the direct and indirect effe ts of alcohol

ra ole was the first compound used in i o (2 3 9), and the ED 50 for the inhibitory effect on the alcohol oxidation in rat was 0 29 to 9 43 mmole/kg (2) The acute oral toxicity (LD 50) in rat was 15 mmole/kg and the minimal lethal dose 12 7 mmole/kg (4) After single doses larger than 15 mmole/kg leading to a comatose state with death occurring within 12 to 36 hours, no liver cell alterations were seen microscopically whereas livers of rats dying 6 to 11 days after the administration of 12 7 to 15 mmole/kg showed extensive centrolobular necroses with inflammatory reactions in the parenchyma and fatty change in surviving liver cells Intraperitoneal administration of pyrazole in rat in a daily dose of 2 2 mmole/kg during 2 to 4 weeks caused anemia and leucopenia, and autopsy revealed mottling of the liver and decrease of the size of the spleen (12) In another experiment in rat (4) pyrazole given daily by stomach tube for 8 weeks, in doses of 0 57 mmole/kg, resulted in retardation of growth but microscopical evidence of liver damage was lacking In another experimental group the rats received the same doses

Received 26 ii 74 Accepted 4 iii 74

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of pyrazole together with alcohol (0 14 to 0 17 mole/kg/day) 76 per cent of the rats died within two weeks and all the rats within 68 days Autopsy revealed liver necrosis in 90 per cent of the rats Pyrazole given to gether with alcohol is thus toxic in doses necessary for the inhibition of LADH This limits the use of this compound in clinical and experimental investigation

4 methylpyrazole is a more potent LADH inhibitor than pyrazole (6, 11) The acute peroral toxicity (LD 50) in rat was found to be 6 5 mmole/kg Daily peronal doses of 1 2 to 2 4 mmole/kg during 4 weeks produced no changes related to treatment (8) Experi ments with tritium labelled 4 methylpyrazole in rat show a rapid conversion into inactive metabolites which are mainly excreted in the urine (1) This may explain the low toxicity of 4 methylpyrazole A dose of 0 084 mmole/kg produces a 70 per cent inhibition of the alcohol oxidation during more than 8 hours (3) There is however, no study published concerning the combined effect of 4 methyl pyrazole and alcohol The use of 4 methyl pyrazole in man is therefore restricted to small doses in order to minimize the risks of unexpected reactions Long term studies with 4 methylpyrazole and alcohol in animals are evidently needed before larger doses can be given to man In the present study in rat 4-methylpyrazole and alcohol have been given by peroral administration during a 12 week period

MATERIAL AND METHODS

4 methylpyrazole was purchased from AB Labkemi (Gothenburg, Sweden)

Male Sprague Dawley rats purchased from AB Anticimex (Sollentuna, Sweden) were housed in individual cages and fed Anticimex Mouse and Rat Food 201 (3 7 per cent fat 37 2 per cent protein) *ad libitum* They had free access to drinking fluid

Totally 48 rats were divided into four groups In the first group which served as control, the rats were maintained on pure water In the second one the drinking water contained 4 methylpyrazole (1 26 mM) and in the third one 4 methylpyrazole (1 26 mM) and alcohol (3 26 M) Finally in the fourth group the water contained alcohol (3 26 M) These solutions were the sole sources of drink ing fluid in the different groups

The rats were 5 6 week old and weighed about 130 g when the experiment commenced The amounts of fluid consumed were registered and the rats were weighed once a week At termination of the experiments, after 12 weeks, the aorta was catheterized under ether anaesthesia Blood sam ples were collected for determinations of hemo globin, leucocytes, differential white cell count, se rum creatinin GPT, GOT, and serum protein Liver, kidneys heart and spleen were weighed and samples of liver, pancreas, kidney and heart were taken for microscopic examination The samples were fixed in 2 per cent paraformaldehyde buffered to pH 7 2 with 0 1 M cacodylate buffer Paraffin embedded sections were stained with haematoxylin eosin and van Gieson's stain Frozen sections were stained for the demonstration of fat with Sudan IV

RESULTS

The rats showed no clinical symptoms as observed in their cages There were no sign of intoxication and their activity seemed un impaired The mean daily doses of alcohol and 4 methylpyrazole, respectively, were cal culated for the three last weeks, and mean values and standard deviation for the different groups are given in Table 1

TABLE 1 *Average Daily Intake of 4 Methylpyra zole (4 MePy) and Alcohol (Alc) during the Last Three Weeks in the Different Groups*

Group	Mean daily dose during the last 3 weeks 4 MePy mmole/kg (mg/kg) body weight	 Alc mole/kg (g/kg) body weight
4 MePy	0 13±0 02 (14 8 ±2 4)	–
4 MePy + Alc	0 10±0 01 (11 7 ±1 7)	0 25±0 04 (11 7 ±1 7
Alc.		0 22±0 03 (10 2 ±1 2)

The mean values for the weight gain in the different groups are illustrated in Fig 1 4 methylpyrazole-treated rats do not differ from the controls while the weight gain in

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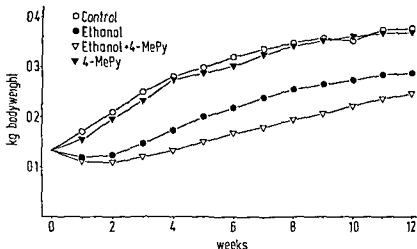


Fig 1 Mean values for the body weights in the different groups registered once a week for 12 weeks

the "4-methylpyrazole + alcohol" group is clearly retarded. The alcohol group takes an intermediate position in this respect. There were no changes in liver function as revealed by the GOT, GPT and serum albumin values, and the results of the haematological investigations revealed no signs of damage to the bone marrow. The renal function was investigated by serum creatinin analyses. The results showed that the serum creatinin in the control group was 0.09 ± 0.02 mmole/l. Two rats in the "alcohol + 4-methylpyrazole" group had serum creatinin values of 0.18 and 0.20, respectively, and one rat in the alcohol group had a value of 0.17 mmole/l.

There were no changes of gross pathology by autopsy. Mean values and S.D. for the weights of total body, liver, kidneys, heart and spleen at autopsy are given for each group in Fig. 2. The organ weights follow the body weights with one apparent exception: the weight of the spleen in the "alcohol + 4-methylpyrazole" group is more depressed than the total body weight (relative weight of the spleen in the controls 0.20 per cent, as compared to 0.17 per cent in the "alcohol + 4-methylpyrazole" group).

Light microscopy revealed no liver changes except varying degrees of excess fat accumulation. The highest degrees of steatosis oc-

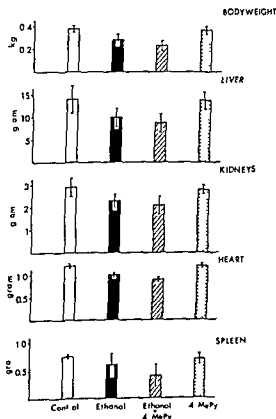


Fig 2 Mean values and S.D. for the body weight, and organ weights at autopsy in the different groups

curred in the alcohol group in which virtually all parenchymal cells contained fat droplets in all but 2 rats There was no preferential localization of the fat within the lobules The fat droplets varied considerably in size and in many instances these droplets were as large as the nuclei Although diffuse occurrence of fat droplets was also observed in half of the "alcohol + 4-methylpyrazole" group, these droplets were regularly fewer and smaller and tended to be most abundant in the centrolobular areas, the remaining rats in this group lacked clear-cut evidence of steatosis in their livers No pathological fat accumulation was noted in the control group, while two out of twelve rats in the 4-methylpyrazole group showed a slight increase of the fat droplets as compared to the controls The only light microscopic change demonstrated in the other organs examined consisted of a round cell infiltration in the heart of a rat in the "alcohol + 4 methylpyrazole" group This is regarded as an accidental finding

DISCUSSION

In this study 4 methylpyrazole was given together with alcohol in doses sufficient to produce a maximal inhibition of the alcohol oxidation during at least 8 hours Opposite to pyrazole (4), 4 methylpyrazole under these circumstances does not induce liver damage as revealed by light microscopy of the liver The increased fat accumulation of the liver cells in this study seems merely related to the presence of alcohol and not to a toxic effect of 4 methylpyrazole The alcohol-induced increase of the fat accumulation decreases when 4-methylpyrazole is present

There is a striking retardation of growth in the rats receiving alcohol compared to the rats in the control group or those receiving 4-methylpyrazole alone The intake of fluid in the alcohol drinking rats is limited by their alcohol oxidizing capacity and, furthermore, alcohol has a diuretic effect This may cause dehydration In addition, the presence of alcohol may decrease the intake of food The retardation of growth is most pronounced in rats receiving alcohol and 4 methylpyrazole

The decrease of the size of the spleen demonstrated in rats after 2–4 weeks' administration of pyrazole, 2 2 mmole/kg/day (12), is not demonstrated in rats receiving similar doses of 4 methylpyrazole for 4 weeks (7) In the present study 0 14 mmole/kg/day of 4 methylpyrazole given with alcohol caused a slight decrease of the relative size of the spleen (see Fig 2) This is difficult to interprete but is unlikely to be related to a toxic reaction

Impaired renal function is reported in the literature after the administration of pyrazole in man (12) Kidney biopsy was not taken in these cases In the present study a moderate increase in the serum creatinin occurred in two rats receiving 4 methylpyrazole and alcohol, and in one rat receiving alcohol alone Kidney biopsy revealed no changes by light microscopic examination The serum creatinin elevation may be due to dehydration caused by the combined effects of restricted intake of the alcohol containing drinking fluid and the diuretic action of alcohol However, it may also be due to a renal damage not revealed by light microscopy

Although the combination of alcohol and 4-methylpyrazole in the doses employed does not produce light microscopic alterations or changes in serum transaminases, the decreased growth rate and signs of impaired renal function makes a continued cautiousness necessary in clinical investigations in man on the effects of 4 methylpyrazole It should also be recalled that light microscopy only reveales cell death, severe cellular degeneration, or fatty change in the living cell Minor toxic effects on the cellular organelles can be best revealed by electron microscopy and refined biochemical investigations Further studies with these methods are obviously needed

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The study has been supported by grants from *The Bank of Sweden Tercentenary Fund* and from *N I A A A*, U S A (A A 00323 01), to Dr *Rolf Blomstrand*

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Acta path microbiol scand Sect A, 82, 539-546, 1974

HISTOLOGICAL CHANGES IN LIVER BIOPSIES FROM PATIENTS WITH CHRONIC HEPATITIS

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A material of 111 biopsies exhibiting chronic hepatitis from 111 patients has been selected from a total consecutive liver biopsy material of 3774 The material is divided into two groups 85 patients exhibiting changes as in chronic aggressive hepatitis and 26 patients with histological changes as in chronic persistent hepatitis The incidence and degree of portal and parenchymal changes in both groups is described A morphologic follow up gives evidence for a general poor prognosis in cases with chronic aggressive hepatitis often with development of cirrhosis, whereas the prognosis in chronic persistent hepatitis is excellent, and cirrhosis will with almost certainty not develop It is stressed that in the cases with chronic persistent hepatitis the morphologic and functional findings only justify the statement, that this condition appears to be chronic persistent hepatitis Only after repeated examinations which reveal no progression, but a similar picture over many months, can the definite diagnosis be made

The term chronic hepatitis has won a permanent place in the hepatologic terminology, but on account of the both clinically and morphologically varying conditions for which the term has been used, there has been and still is to a certain degree considerable confusion

While there are several investigations concerning which morphological changes are seen in liver biopsies from selected groups of patients with chronic hepatitis (2, 10, 11), no material exists, to the best of the authors' knowledge, which gives information on which changes are seen in consecutive materials of biopsies from patients with chronic hepatitis

Received 7 xii 73 Accepted 7 iii 74

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The purpose of the present paper has been to describe the incidence and the degree of different histological qualities in a consecutive series of liver biopsies from patients from a Scandinavian metropolis with chronic hepatitis as defined by *de Groote et al* (5) Furthermore, a preliminary follow-up has been performed

MATERIAL AND METHODS

The material consists of 111 percutaneous liver biopsies from 111 consecutive patients all with histologically verified chronic hepatitis (5)

The diagnosis of *chronic aggressive hepatitis* is based on the morphological criteria of *de Groote et al* (5) The portal tracts are moderately to severely enlarged partly on account of development of connective tissue and bile duct proliferation and partly, and chiefly, on account of heavy infiltration with inflammatory cells dominated by lympho-

cytes, plasma cells and histiocytes The inflammatory infiltration extends into the peripheral portions of the lobules, especially in relation to larger or smaller piece meal necroses which are a conspicuous and constant finding

The diagnosis of *chronic persistent hepatitis* is made by relating clinical and biochemical findings in cases of long standing liver affection with portal changes in the shape of slight enlargement on account of a rather dense infiltration with inflammatory cells, chiefly lymphocytes and histiocytes, possibly with a number of plasma cells

There is no or only slight fibrosis, and in principle the portal tracts are well demarcated with an intact limiting plate, though a single smaller piece meal necrosis occasionally may be seen

Cases morphologically giving suspicion of cirrhosis have been excluded and so have cases with acute viral hepatitis with slight or moderate periportal changes in the form of piece meal necroses

The material has been obtained as consecutive biopsies from patients with long standing liver affection and with histologically verified chronic hepatitis admitted to Medical Department 2, Kommunehospitalet, Medical Department B, Bispebjerg Hospital and The University Clinic for Infectious Diseases, Blegdamshospitalet, Copenhagen during the period December 1965 August 1972 In the same period a total of 3774 percutaneous liver biopsies were received from the same departments at the Pathological Anatomical Institute, Kommunehospitalet

The liver biopsies have been performed by the method of Menghini and the tissue has been fixed in neutral formalin and imbedded in paraffin The biopsies are 1 1 4 mm thick and 1 5 4 5 cm long and are cut on a rotary microtome (6 μm 50 sections)

The assessment has been performed by one of the authors (P C) on haematoxylin and eosin and v Gieson Hansen preparations For all biopsies further sections, stained for reticulin fibres (7), iron (8) and pyroninophil substance (3) have been available

The following qualities have been semiquantitatively registered (0, +, + +, + + +) focal necroses confluent necroses, piece meal necroses, variation in size of liver cells and nuclei acidophilic bodies, Kupffer cell proliferation cholestasis (non controversial intra and extracellular bile thrombi), adenomatous liver cell proliferation fatty change, parenchymal inflammation, passive septa (1), as well as iron and lipofuscin deposits in liver cells, Kupffer cells, and connective tissue

Further, the degree of bile duct proliferation fibrosis, and inflammation in portal tracts has been registered Also the occurrence of bile ducts with abnormal epithelium has been recorded (9)

On 83 patients one or more repeat biopsies have been performed, and from 38 patients one or more earlier biopsies are available These biopsies have been assessed according to the same criteria as the above mentioned

RESULTS

85 of the biopsies (77 per cent) fulfill the histological criteria for chronic aggressive hepatitis, while 26 of the biopsies (23 per cent) show portal changes consistent with chronic persistent hepatitis

Chronic Aggressive Hepatitis

A summary of the *parenchymal changes*

TABLE 1 *Number of Biopsies with the Following Parenchyma Changes from 85 Patients with Chronic Aggressive Hepatitis*

Parenchymal changes	0	+	+ +	+ +
Focal necroses		79	6	
Confluent necroses	62	19	4	
Passive septa	57	26	2	
Piece meal necroses		35	49	1
Acidophilic bodies	8	70	7	
Variation of cells and/or nuclei	2	57	26	
Fatty change	64	21		
Kupffer cell proliferation		73	10	2
Adenomatous proliferation	79	6		
Cholestasis	76	9		
Parenchymal inflammation	23	62		
Lipofuscin in liver cells		82	3	
Iron in liver cells	82	1	2	
Iron in Kupffer cells	71	9	5	

in the 85 biopsies with chronic aggressive hepatitis is given in Table 1

Focal necroses may be found in all areas of the parenchyma, but are, however, most frequent in the central part of the lobule

Confluent necroses are most often small and in the centrilobular zone of the lobule Confluent necroses and *passive septa* are found in the same biopsy in 20 cases

The periportal areas always exhibit more or less pronounced destruction of the limiting plate with many closely packed, often rather small areas of *piece-meal necroses*

Acidophilic bodies are, just as focal necroses, most often found in the centrilobular zone

Adenomatous proliferation is less frequent than cholestasis and usually a bile thrombus could be identified in the small lumen

Mesenchymal reaction in the parenchyma All the biopsies exhibit some degree of *Kupffer* cell proliferation In two cases there is severe and in ten cases a moderate proliferation The degree of inflammation in all 62 biopsies with inflammatory cells is slight Lymphocytes are encountered in 51 biopsies while plasma cells are found in 55 cases Neutrophils have only been demonstrated in a total of two biopsies and eosinophils in a total of one

A summary of the changes in *the portal tracts* is given in Table 2

Inflammation All biopsies show portal inflammation Lymphocytes macrophages and plasma cells are demonstrated in all biopsies The predominant inflammatory cell in the periportal area is the plasma cell and as a rule their number is so high that this area appears as a red zone in pyronine stained sections The number of biopsies with neutrophils is 48, with eosinophils 18

Abnormal bile duct epithelium is found in 28 (33 per cent) of the 85 biopsies The main histologic changes observed in the affected portal bile ducts are most often multilayered epithelium with swollen cells with pale, lightly coloured, often "empty looking" or vacuolated cytoplasm Often the cells are partly confluent with more or less ill-defined cellular limits The nucleus may show karyopyknosis and/or karyorrhexis Generally there are areas with an indistinct basement membrane The bile duct epithelium and the basement membrane are frequently infiltrated with lymphocytes and sometimes plasma cells and neutrophils The lumen may contain cellular debris and may be partly obliterated

Germinal centers are seen in two cases and are in both biopsies found close to the parts of the biliary tree exhibiting abnormal epithelium

Chronic Persistent Hepatitis

A summary of the *parenchymal changes* in the 26 biopsies with chronic persistent hepatitis is given in Table 3

A few *focal necroses*, distributed diffusely throughout the parenchyma, are found in most biopsies whereas *confluent necroses* and *passive septa* are not observed in any of the cases

Mesenchymal reaction in the parenchyma All the biopsies exhibit a slight degree of Kupffer cell proliferation Two cases in addi-

TABLE 2 *Number of Biopsies with the Following Portal Changes from 85 Patients with Chronic Aggressive Hepatitis*

Portal changes	0	+	+ +	+ + +
Fibroses		48	37	
Inflammation		2	32	51
Bile duct proliferation		48	35	2
Abnormal bile duct epithelium	57	28		
Iron in portal macrophage	69	8	8	–
Germinal centres	83	2		

TABLE 3 *Number of Biopsies with the Following Parenchymal Changes from 26 Patients with Chronic Persistent Hepatitis*

Parenchymal changes	0	+	++	+++
Focal necroses	3	23	–	–
Confluent necroses	26	–	–	–
Passive septa	26	–		–
Piece meal necroses	23	3	–	–
Acidophilic bodies	21	5	–	–
Variation of cells and/or nuclei	5	21	–	–
Fatty change	24	2	–	–
Kupffer cell proliferation	–	26	–	–
Adenomatous proliferation	26	–	–	–
Cholestasis	26	–	–	–
Parenchymal inflammation	24	2	–	–
Lipofuscin in liver cells	–	26	–	–
Iron in liver cells	24	1	1	–
Iron in Kupffer cells	25	1	–	–

TABLE 4 *Number of Biopsies with the Following Portal Changes from 26 Patients with Chronic Persistent Hepatitis*

Portal changes	0	+	++	+++
Fibroses	2	23	1	–
Inflammation	–	10	14	2
Bile duct proliferation	15	9	2	–
Abnormal bile duct epithelium	24	2	–	–
Iron in portal macrophages	25	–	1	–
Germinal centres	26	–	–	–

tion contain a few lymphocytes in the parenchyma Plasma cells and neutrophils have not been demonstrated in the parenchyma

A summary of the changes in the *portal tracts* is given in Table 4

All biopsies show *portal inflammation* Lymphocytes and macrophages are demonstrated in all cases and plasma cells in 25 biopsies The number of biopsies with neutrophils is five, and with eosinophils two

Abnormal bile duct epithelium is found in two of the 26 biopsies and the same two cases exhibit the most pronounced portal inflammation

Preliminary Follow up

Chronic aggressive hepatitis Table 5 shows the distribution according to chief histological diagnosis of the latest of the repeat biopsies from 67 of the 85 patients with chronic aggressive hepatitis The average period of observation has been 20 months, varying from one to 60 months

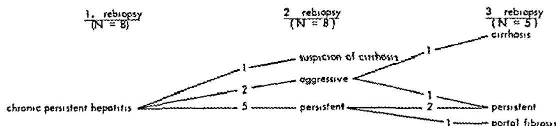
26 cases (39 per cent) developed cirrhosis, and in two cases there is suspicion of cirrhosis 20 cases still show chronic aggressive hepatitis, whereas 19 biopsies showed histologic improvement

In 22 of the 67 patients with repeat biopsies (33 per cent) bile ducts with atypical epithelium were demonstrated in the primary biopsies 13 of these cases developed cirrhosis, five still show chronic agressive hepatitis, three show chronic persistent hepatitis and one portal fibrosis

In the repeat biopsies abnormal bile duct epithelium was demonstrated in an additional 8 patients (three of the cases with cirrhosis, one of the cases with suspicion of cirrhosis three of the cases with chronic aggressive hepatitis and one of the cases with chronic persistent hepatitis)

Two ore more repeat biopsies are present

TABLE 7 *The Table Shows the Distribution of Repeat Biopsies According to Histological Diagnosis in 8 Patients with Chronic Aggressive Hepatitis in Whom the first Rebiopsy Showed Histological Changes as in Chronic Persistent Hepatitis*



fluent necrosis and five with abnormal bile duct epithelium) 23 of these are included in the temporary follow-up Six developed cirrhosis, nine still have chronic aggressive hepatitis, five have chronic persistent hepatitis, and three portal fibrosis The average period of observation from the first biopsy showing acute viral hepatitis to the first showing chronic aggressive hepatitis has been 6 months varying from 1 to 23 months

From 13 of the 26 patients registered as chronic persistent hepatitis earlier biopsies are available All of these show acute viral hepatitis Eight of these are included in the temporary follow-up Four still show chronic persistent hepatitis, one has developed cirrhosis, two portal fibrosis, and one shows liver without pathological changes The average period of observation from the first biopsy exhibiting acute viral hepatitis to the first showing chronic persistent hepatitis has been 9 months varying from 2 to 26 months

TABLE 8 *The Table Shows the Distribution of the Latest of the Repeat Biopsies According to Histological Diagnosis in 16 Patients with Chronic Persistent Hepatitis The Average Period of Observation has been 10 Months Varying from one to 32 Months*

Chief histological diagnosis	
Cirrhosis	1
Chronic aggressive hepa i	1
Chronic persistent hepatit	10
Liver with portal fibrosis	3
Liver without pathologic atures	

DISCUSSION

As it appears from the results, chronic hepatitis is found in biopsies from 111 patients out of the 3774 biopsies examined from three medical departments It must be noted that the figures given are minimum values, since all cases which morphologically give suspicion of cirrhosis have been excluded It must therefore be justifiable to claim that chronic hepatitis in our material which is derived from a Scandinavian metropolitan area is not exceedingly rare

The only differential diagnostic possibilities are cirrhosis and acute hepatitis with suspicion of chronicity In biopsies from patients with cirrhosis a histological appearance is sometimes seen which only with difficulty may be discerned from chronic aggressive hepatitis This is seen in macronodular cirrhosis developing from different kinds of hepatitis The changes are irregularly distributed with regeneration nodules of varying size alternating with areas with partly conserved lobular architecture Reticulin stained sections and serial sections are as a rule of considerable value in cases of this kind

Acute hepatitis with suspicion of chronicity has considerable morphologic resemblance to chronic aggressive hepatitis with signs of acute hepatitis and there is often a gradual transition between the two conditions For the diagnosis chronic hepatitis with signs of viral hepatitis support may be obtained by observing the changes in the portal areas where especially the increase in connective tissue and the proli

TABLE 5 *The Table Shows the Distribution of the Latest of the Repeat Biopsies with Regard to Histological Diagnosis in 67 Patients with Chronic Aggressive Hepatitis The Average Period of Observation has been 20 Months Varying from One to 60 Months*

Chief histological diagnosis	
Cirrhosis	26
Suspicion of cirrhosis	2
Chronic aggressive hepatitis	20
Chronic persistent hepatitis	12
Liver with portal fibrosis	7

from 34 of the 85 patients with chronic aggressive hepatitis The distribution according to histological diagnosis of the first, second and third repeat biopsy is given in Table 6 In 10 of these 34 patients the first repeat biopsy showed cirrhosis, one gave suspicion of cirrhosis, and three cases exhibited portal fibrosis These diagnosis were upheld in the following biopsies from these patients

In 12 of the 34 patients the first repeat biopsy showed chronic aggressive hepatitis In the second repeat biopsy 9 cases still showed chronic aggressive hepatitis, while two cases exhibited changes as in chronic persistent hepatitis, and one showed transition into cirrhosis From four patients (three with chronic aggressive and one with chronic persistent hepatitis) a third repeat biopsy is available Two of these exhibit changes as in chronic persistent hepatitis One continues to exhibit chronic persistent hepatitis and one shows cirrhosis

In eight of the cases with more than one repeat biopsy the first repeat biopsy showed chronic persistent hepatitis Table 7 indicates chief histological diagnosis on second and possible third repeat biopsy from these 8 patients On the second repeat biopsy 5 patients continued to show changes as in chronic persistent hepatitis, and 3 exhibited an exacerbation of the histological changes (one suspicion of cirrhosis, and two chronic aggressive hepatitis) The latter two patients in a third biopsy showed cirrhosis and chronic persistent hepatitis respectively, while the third rebiopsy from three cases with chronic persistent hepatitis show unaltered changes in two cases and histological improvement in one case

Chronic persistent hepatitis Table 8 shows the distribution according to chief histological diagnosis of the latest of the repeat biopsies from 16 of the 26 patients with chronic persistent hepatitis The average period for observation has been 10 months varying from 1 to 32 months One case developed cirrhosis, and one chronic aggressive hepatitis Ten cases still show chronic persistent hepatitis, whereas four biopsies showed histological improvement

Abnormal bile duct epithelium was not demonstrated in any of the original biopsies from these 16 patients, but the repeat biopsy showing chronic aggressive hepatitis in addition exhibited abnormal bile duct epithelium

Retrospective study From 25 of the 85 patients with chronic aggressive hepatitis one or more earlier biopsies are available They all exhibited acute viral hepatitis (4 with con-

TABLE 6 *The Table Shows the Distribution of 1, 2 and 3 Repeat Biopsy According to Histological Diagnosis in 67 Patients with Chronic Aggressive Hepatitis The First Numbers are Cases with Unaltered Diagnosis between two Biopsies*

	Primary biopsy (N = 85)	1 rebiopsy (N = 67)	2 rebiopsy (N = 34)	3 rebiopsy (N = 16)
Cirrhosis		23	10 + 1	4 + 2
Suspicion of cirrhosis		1	1 + 1	1 + 0
Chronic aggressive hepatitis	85	26 + 0	9 + 2	0 + 0
Chronic persistent hepatitis		12	5 + 2	3 + 3
Liver with portal fibroses		5	3 + 0	2 + 1

ducts is more pronounced than in acute hepatitis

In general the distinction between chronic aggressive and chronic persistent hepatitis, using the histological criteria as given by De Groote et al (5), presented no problem

Chronic aggressive hepatitis is characterized by inflammatory infiltration of portal tracts and adjacent parenchyma and by necroses of hepatocytes in the limiting membrane (piece meal necrosis) In addition there are portal fibroses and some degree of bile duct proliferation

The lobule regularly shows slight to moderate changes as in viral hepatitis About one fourth of the cases show areas with confluent necroses These areas are often small and most frequently localized to the centrilobular zone Approximately one fourth contain so called passive septa 54 of the cases neither show confluent necroses nor passive septa This in connection with the fact that only four of the 25 cases with acute viral hepatitis which later developed chronic aggressive hepatitis show confluent necroses speaks in favour of other factors being causative in the development of chronic liver disease in patients with acute viral hepatitis (2)

It further appears that approximately one third of the biopsies with chronic aggressive hepatitis concurrently exhibit atypical bile duct epithelium

Transition from acute viral to chronic aggressive hepatitis may be suspected if the histological features of acute hepatitis are accompained by a disproportionate degree of portal inflammation extensive necrosis at the junctions between portal tract and lobule and portal fibroses The transition may be considered proven when a sequency of biopsies show gradual transition from typical viral hepatitis with no signs of chronicity to typical chronic aggressive hepatitis with morphological features as described above In the present material about one third (29 per cent) had an initial biopsy performed These biopsies exhibited in all cases a typical acute viral hepatitis

In the rest of the material the disease develops insidiously, and it is predominantly in this part of the material that abnormal bile duct epithelium occurs In a previous study (4) it was shown that in the group of chronic aggressive hepatitis with abnormal bile duct epithelium there was more pronounced preponderance of women, a more frequently insidous development, and a higher incidence of organ non specific antinuclear factors than in the group of chronic aggressive hepatitis without abnormal bile duct epithelium

There is thus evidence for the existence of more than one cause of chronic aggressive hepatitis *Wright* (13) reported that patients with active chronic hepatitis with Au antigen in their serum had a lower titre of smooth muscle antibodies than those without, and in patients studied by *Vischer* (12) Au antigen and smooth muscle antibodies were mutually exclusive These observations suggest that one type of chronic aggressive hepatitis may be initiated by a viral infection with Au antigen and debut as an acute viral hepatitis, while another type may be an autoimmune disease The relevance of this serological classification has previously been reported (6)

As it appears from the results about one third (33 per cent) of the patients with chronic aggressive hepatitis developed cirrhosis It must be noted that the figures given are minimal values since the follow up is only preliminary at the present time

Chronic aggressive hepatitis with abnormal bile duct epithelium carries a poorer prognosis than the same condition without abnormal bile ducts (4), and it is noteworthy that while one third of the patients present abnormal bile duct epithelium in the primary biopsy about two thirds of the patients which develop cirrhosis have abnormal bile duct epithelium in one or another of the biopsies

In about one fourth the follow up shows histological improvement The cases with histological signs of chronic persistent hepatitis in the last biopsy may as shown by the follow up analysis (Table 7) be a variant of the histological lesion in patients with

chronic agressive hepatitis Only the seven cases with portal fibroses thus show a significant improvement, and as at least three of these cases have debuted as acute viral hepatitis, it is possible, that this group, or at least part of it, does not represent genuine chronic aggressive hepatitis It must therefore be reasonable to claim that patients with chronic aggressive hepatitis in a consecutive material often develop cirrhosis and that the prognosis is, in most instances, poor

In chronic persistent hepatitis the inflammation, unlike that of chronic aggressive hepatitis, is virtually confined to the portal tracts The limiting plates are intact, and parenchymal cell damage is usually slight Symptoms tend to be indefinite, and it is often difficult to be sure whether chronic persistent hepatitis represents a distinct disease process, or whether it is merely a variant of the histological lesion of late viral hepatitis or chronic aggressive hepatitis

Half of the patients had an intitiating attack clinically like acute viral hepatitis and biopsy at the time in all cases showed typical acute viral hepatitis The evidence indicates *that chronic persistent hepatitis may often,* though not always be a sequel to acute viral hepatitis

The generally benign course of chronic persistent hepatitis is demonstrated by the follow-up and, as mentioned above, it is probable that the two cases which developed cirrhosis and chronic aggressive hepatitis do not represent genuine chronic persistent hepatitis

The morphological and functional findings only justify the statement that this condition appears to be chronic persistent hepatitis Only after repeated examinations which reveal no progression but a similar picture over many months can the definite diagnosis be made

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LARYNGEAL LESIONS IN URBACH-WIETHE DISEASE (LIPOGLYCOPROTEINOSIS; LIPOID PROTEINOSIS; HYALINOSIS CUTIS ET MUCOSAE)

A Histopathological and Clinical Study, Including Direct Laryngoscopical Examinations

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In Northern Sweden 27 patients with Urbach Wiethe disease (lipoglycoproteinosis, lipoid proteinosis, hyalinosis cutis et mucosae) are known Twenty-six of them were hoarse and one was not According to anamnesis, the hoarseness had been present since the first years of life. Some earlier diagnoses indicate that the disease may be confused with more common disorders such as chronic laryngitis and singer s nodule In agreement with preceding reports it was observed in 4 of our patients that the laryngeal changes in UWD may be demonstrated by tomography In one of the Swedish patients the laryngeal symptoms necessitated a tracheostomy The impression from a laryngoscopical study of 10 patients was that the laryngeal changes are more conspicuous in patients with more pronounced hoarseness The hoarseness seems to be due to air leakage, as perfect closure of the vocal cords is impossible due to their irregular borders A histopathological study of 7 patients showed that the vocal cords may contain abundant deposits of the substance typical for UWD and that deposits can extend throughout the whole submucous layer down to the muscular coat The UWD deposits were stained yellow in van Gieson s and Herovici s methods and red with haematoxylin and eosin A positive reaction was obtained with the periodic acid Schiff procedure and to a moderate degree also with the p-dimethylaminobenzaldehyde reaction for tryptophan and a negative reaction for fibrin was obtained with the phosphotungstic acid method Unequivocally positive reactions were not obtained with the methods for amyloid The reticulin staining procedure gave variable results It was found that differentiation between degenerated elastin and UWD deposits may at least sometimes be difficult unless a specific method for elastin is applied

Urbach Wiethe disease (UWD), also called lipoglycoproteinosis lipoid proteinosis or hyalinosis cutis et mucosae affects the skin and the mucous membranes of the mouth pharynx and larynx (cf *Hofer* 1973) The lesions may be rather inconspicuous However, the most characteristic symptom in UWD is a chronic hoarseness, apparently due to laryngeal lesions This hoarseness is usually said to have been present since the first years of life and sometimes even at birth UWD is reported to be recessively inherited Somewhat

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more than 200 cases have been described

Clinically, there may be discrete, diffuse or nodular infiltrates in the skin and also acne-like scars The latter, if present, may appear even outside typical acne-regions The most characteristic cutaneous alteration is the occurrence of small papular lesions, each usually not more than one millimeter in diameter, along the free borders of the eyelids (cf *Hofer* 1973) Affected mucous membranes may become whitish or yellowish in colour and their surfaces may be papular or in other ways slightly irregular in many regions of the mouth, pharynx and larynx Besides the hoarseness, frequent characteristic findings are a thickening of the lower lip and thickening and diminished motility of the tongue On the back wall of the pharynx there may be whitish or yellow white patches, points, or net work (cf *Hofer* 1973) It has previously not been emphasized that this also seems to represent a frequent and easily observable lesion (*Öhman* & *Hofer*, unpublished)

Histopathologically, there are *in skin and* affected mucosal regions, deposits of an amorphous substance, in distribution suggestive of amyloidosis but usually not stained at procedures considered to be specific for amyloid The deposits are PAS positive and often sudanophil The initial lesion seems to be a thickening of the walls of small blood vessels a pattern resembling diabetic microangiopathy (*Hofer* 1973)

In Northern Sweden 27 UWD patients are known at present Apart from the early report of 6 patients by *Wiethe* (1927), most previous publications in the otorhinolaryngological literature on UWD concern only single patients (*Hofer* 1973) The purpose of the present investigation was to study the laryngeal lesions in UWD from anamnestic, laryngoscopical and histopathological points *of view in order to obtain a better under*standing of their pathogenesis and their sequelae Detailed case histories in these respects have been obtained from most of the patients from Northern Sweden Direct laryngoscopical examination if possible in combination with photographic documentation, was performed in about 10 patients by the same examiner (J Ö) In 7 cases biopsy material from the larynx was available for microscopic investigation

MATERIAL AND METHODS

Past History

The sex and year of birth of the 27 UWD patients in this series are shown in Table I The patients have been recorded with a sibship number *in Roman numerals (in two cases combined with* a small "a")—followed by an Arabic numeral if the sibship contains more than one patient (cf *Hofer* 1974)

Some data, including information about preceding indirect and direct laryngoscopy, have for most of the 27 patients been obtained from earlier hospital and out patient records Most patients have also been interviewed about their symptoms

Four of our patients were examined by tomography A few preceding reports (*MacKinnon* 1968, *Weidner et al* 1970) indicated that the laryngeal changes in UWD may be demonstrated by this roentgenographic procedure

Otorhinolaryngological Examination

Table I indicates the degree of hoarseness of most patients Conventional otorhinolaryngological examination was performed in 10 of them (Tables I and 2) Suspension laryngoscopy with photo-documentation was used in 8 of them according to the *Kleinsasser* (1968) technique The patients were intubated under general anesthesia supplemented by muscle relaxants In view of the purpose of this report, only the laryngeal findings will be described

Histopathological Examination

The histopathological diagnosis of UWD can be established in biopsies from skin or from non laryngeal mucous membranes, for example in biopsies from the mucosa of the lower lip Therefore, in the present study it was considered unnecessary to take biopsy specimens from the vocal cords However, previously taken biopsy specimens from the vocal cords, fixed in formalin and embedded in paraffin, were available from several of the patients listed in Table 1, *viz* from patient VIII 1 from 1968 and from 1957, from his brother [illegible] VIII 4) from [illegible] ient IV [illegible] nd from [illegible] biopsy specimens were stained according to van Gieson's method, with the van Gieson-elastin stain, by the periodic acid Schiff (PAS) procedure, and with

Herovici's (1963) variant of van Gieson's method If available paraffin embedded specimens contained more than insignificant amounts of connective tissue stroma, the following staining methods were also applied conventional haematoxylin and eosin stain, alkaline Congo red method (*Puchtler et al* 1962) for amyloid, Thioflavin T (*Vassar & Culling* 1959) for amyloid, p dimethyl aminobenzaldehyde (DMAB) reaction (*Adams* 1957) for tryptophan, phosphotungstic acid haematoxylin (PTAH) method for fibrin and Laidlaw's silver impregnation method for reticulin Sections stained with alkaline Congo red were examined both in direct transmitted illumination and in polarized light (*Missmahl & Hartwig* 1953) Sections stained with Thioflavin T were examined in a fluorescence microscope equipped with a super pressure mercury lamp HBO 200 W, in epi illumination and with exciter filter BG 12 and barrier filter combination 53 + 44 Frozen sections were cut from part of the material from 1968 from patient VIII 1 and stained with a red Sudan dye

RESULTS

Past History

First appearance of hoarseness One adult patient, with otherwise quite characteristic UWD symptoms, was not at all hoarse (Table 1) In the remaining 26 cases it was stated by the patients themselves or by their parents that the hoarseness or weak voice had been present since birth or from the first years of life Some of the patients had been examined by a physician because of their peculiar voice during the first years of life For example, one patient was said to have a lesion suggestive of singer's nodule on one vocal cord at the age of 20 months and in another patient the true vocal cords were said to be thick and the false vocal cords to be even thicker at the age of 30 months

Changes in the degree of hoarseness Most patients stated that the hoarseness had remained relatively unaltered over the years a few said that it had been worse when they were young and a few others that it had increased with increasing age In patients VII and X the hoarseness was said to have diminished at puberty Some patients became more hoarse in damp weather Some patients but not all, developed increased hoarseness when smoking or when drinking alcoholic beverages A few stated that the voice became somewhat better after a small amount of alcohol because they then became more relaxed A few of the female patients said that the hoarseness increased during pregnancy With few exceptions, it was stated that no kind of food, childhood disease, medicine or vaccination had had any influence on the degree of hoarseness

Previous clinical diagnoses It is obvious that the laryngeal findings can easily be misinterpreted and consequently, the correct diagnosis may be missed by a physician unfamiliar with UWD Thus, the diagnosis on one or several occasions for 6 of the patients from Northern Sweden was "chronic laryngitis" or "chronic laryngitis with acute exacerbation" Five patients had on one or more occasions, although consulting the doctor for their hoarseness, been described as having normal vocal cords, in one patient even after direct laryngoscopy Other diagnoses have been "fibroma laryngis", 'aphonia (congenital?)", 'chondritis laryngis' and "singer's nodules"

In 4 patients the hoarseness was apparently considered to be due to overstrain of the vocal cords, as the diagnoses were "functional hoarseness" 'transversus paresis" and 'internus paresis" Patient VIII 1, in whom the right vocal cord was later extirpated due to stiffness caused by deposits, was diagnosed as having 'paralysis of the right vocal cord' as early as 1951

Some of the patients who on one or several occasions had been described as having normal vocal cords had on other occasions been said to have somewhat oedematous or somewhat thick vocal cords For patient IX, born 1950, the laryngoscopical findings on various occasions were reported as follows "normal' (1953), "internus paresis" in the left vocal cord (1959), "normal' (1962), and "vocal cords somewhat thick' (1965) Three patients were said to use false vocal cords in intonation

Tomographical examination The laryngeal lesions may be illustrated radiographically Thus in the 4 cases where an X-ray exami-

TABLE 1 *Data about the 27 known UWD Patients from Northern Sweden*

Patient	Sex	Year of birth	Degree of hoarseness*	Description of laryngoscopical findings given in the text	Description of histopathological findings given in the text and/or in Tables 3 and 4
I 1	F	1924	s m	+	
I 2	M	1936	s		
I 3§	M	1939	s m ?		
II	M	1905	m p		+
IIa	F	1961	m		
III 1†	F	1899			
III 2	M	1906	m	+	
IV	F	1942	s m	+	+
V 1	M	1910	m		
V 2	M	1920	s m		
V 3	M	1932	0		
VI 1	F	1944	p	+	+
VI 2	F	1948	m	+	
VII	M	1921	m	+	+
VIII 1$	M	1903		+	+
VIII 2	M	1905	m p		+
VIII 3	F	1907	m	+	+
IX	M	1950	p	+	
X	M	1955	s	+	
XI	F	1962	p		
XII	F	1961	m p		
XIIa	F	1927	m		
XIII 1	F	1918	s		
XIII 2	F	1922	p		
XIII 3	M	1930	m		
XIV 1	F	1940	m		
XIV 2	F	1945	s		

* Degree of hoarseness is designated s = slight, m = moderate p = pronounced 0 = no hoarseness
§ Degree of hoarseness difficult to evaluate as the patient who had Down's syndrome, hardly spoke at all
† The patient was hoarse, but degree of hoarseness not evaluated
$ One vocal cord extirpated and reliable information about degree of hoarseness before that operation was not obtained

nation with tomography had been performed previously the findings were as follows III 1 irregular and thick vocal cords and false cords somewhat defective closure of the true vocal cords which show somewhat limited motility VIII 1 thickened false and true vocal cords insufficient motility at intonation and respiration VIII 2 signs of oedema in larynx particularly in the sub glottic region the position of the vocal cords is compatible with a "posticus paresis" XIII 2 both false vocal cords thick, swollen particularly on the right side with filling of Morgagni's sinus, at intonation both vocal cords participate in closure but Morgagni's sinus becomes entirely filled by the swollen false cords At respiration the left false cord returns to a normal position but on the right side there is still thickening distally

Laryngoscopical Findings

Table 1 shows that in 26 patients all degrees of hoarseness were present from slight to pronounced As already mentioned, one patient was not hoarse The laryngoscopical

TABLE 2 *Laryngoscopical Observations in Urbach Wiethe Disease*

Patient	Lesions observed in			
	epiglottis	false vocal cords	true vocal cords	subglottic region
I 1	–	–	+	–
III 2	–	–	+	–
IV	+	+	+	
VI 1	–	–	+	–
VI 2	–	–	+	–
VII	–	–	+	+
VIII 1	–	–*	+	–
VIII 3	–	–	+	+
IX	+	+	+	–
X	–	–	+	–

Symbols used are + characteristic UWD changes present, – changes of UWD type absent
* Concerns the latest examination, the thickening of one false vocal cord on a previous occasion may perhaps have been related to an acute laryngitis

findings in 10 patients, described in detail below, are summarized in Table 2

Patient VIII 1 In November 1963 indirect laryngoscopy showed the vocal cords to be irregularly thickened with yellow white deposits A notable fact is that as early as 1951 the right vocal cord had been observed to be relatively immobile In 1963 gradually increasing stridor made it necessary to perform a tracheostomy In 1968 a right sided laterofixation operation was carried out but failed to benefit the patient Therefore, a laryngofissure operation with extirpation of the right vocal cord was performed the same year This operation resulted in considerable widening of the airway and the patient could soon be decanylated

Patient VIII 3 Both vocal cords were yellowish due to deposits Particularly the right vocal cord

TABLE 3 *Summary of Some Histopathological Findings in Vocal Cord Specimens from Patients with Urbach Wiethe Disease, Described in Detail in the Text*

pecimen from patient	Deposits found				Degeneration in elastic fibres present	Oedema present	Inflammatory cells present
	in vessel walls	in connective tissue outside vessel walls	around excretory duct of mucous gland	around superficial striated muscle cells			
left)	+	+	/	/	(+)	+	+
right)	+	/	/	/	/	+	+
	+	+	+	(+)	+	+	+
I*	+	/	/	/	/	/	/
§	+	/	/	/	/	/	/
I 1 (1957)	+	+	(+)	/	+	/	(+)
I 1 (1968)	+	+	+	+	+	–	–
I 2	+	/	/	/	/	/	+
I 3	+	+	/	/	+	/	–

Symbols used are + indicated alteration present, (+) somewhat questionable whether indicated alteration present, – indicated alteration absent, / available specimen not possible to judge in that respect.
* UWD deposits demonstrable, although only minute amounts of subepithelial tissue present in the small biopsy specimens (left vocal cord)
§ Thickened vessel walls indicating presence of deposits but detailed analysis impossible as the biopsy specimen (right vocal cord) had obviously been exposed to a too high temperature during the embedding procedure



Fig 1 Patient IX *Photograph of the vocal cords* The borders of both vocal cords are affected by irregular yellowish white deposits The vocal cords are seen from an angle at which the deposits (white in the black and white photograph) are most prominent in the middle part of the right vocal cord Kleinsasser technique

had a wavy surface That, in combination with a subglottic lesion, gave rise to slits with leakage of air at both commissures The false vocal cords did not seem to be affected

Patient I I Both vocal cords, particularly the left one, were thick, yellowish-white, and irregular, apparently due to deposits

Patient III 2 The left vocal cord was slightly granular Both vocal cords were somewhat flaccid The epiglottis was *normal*

Patient IV The true vocal cords had the characteristic yellowish white deposits also observed in several of the other patients The left vocal cord was particularly affected The posterior halves of the vocal cords were most affected The lesions resulted in thickening *of the vocal cords, particularly* the left one which also had the most uneven border There was a small region with air leakage posteriorly but no observable reduction in the motility of the vocal cords The epiglottis and the false vocal cords showed only discrete lesions

Patient I I I In 1972 it was found that the vocal cord were *yellowish white, thick and with* uneven bor r giving rise to large air slits during phonation rtunately the mouth was too narrow to acce fairly large laryngoscope, and photodocume was therefore not possible No additional lar nges were observed

Patient VI tility of the vocal cords was normal T *somewhat more yellowish* than normal an u places they were slightly thickened due to re rely inconspicuous deposits

Both vocal cords had wavy free borders more marked on the right vocal cord, apparently giving rise to air leakage and hoarseness

Patient VII Both vocal cords, particularly the left one, were thickened due to yellowish deposits The free border of the left vocal cord showed a rather markedly uneven contour and the clinical impression was that there was air leakage *posteriorly* There seemed to be a slightly larger, yellowish *deposit in the anterior commissure just below the* level of the vocal cords The epiglottis was omega shaped but showed no other changes

Patient IX The true vocal cords were markedly thicker than normal with yellowish white deposits, particularly in the middle region of the right vocal cord (Fig 1) Similar but less conspicuous changes were also seen in the most anterior part of the false vocal cords The motility of the true vocal cords was normal The mucous membranes of the false vocal cords were reddened and somewhat dry The epiglottis was slightly thickened and deformed with two small whitish yellow deposits on the border of the epiglottis

Patient X The true vocal cords were slightly thicker and slightly more uneven than normal but more characteristic deposits were seen only just above *the region of the right arytenoid cartilage* The motility of the vocal cords was normal The epiglottis and the false vocal cords appeared to be normal It may be noted that this patient, who had only these subtle laryngeal changes, also had only a slight degree of hoarseness (Table 1)

Histopathological Findings

Some of the histopathological findings are summarized *in Table 3 As little is known* from previous literature about histopathological alterations in vocal cords in UWD patients details for some of the specimens are given below

Patient VIII 1 In the extirpated vocal cord there were abundant amounts of the picrinophil PAS positive substance characteristic for UWD (cf *Hofer* 1973) in the broad connective tissue layer between the surface epithelium and the layer of striated muscle (Fig 2) In a relatively large area deposits almost totally replaced the normal tissues. Outside this area, in the connective tissue layer, there was not only thickening of the walls of small blood vessels, most of them with narrow lumina, but there were also deposits in the form of smaller, extravascular, indistinctly demarcated patches, most of them included in a relatively superficially located band The excretory duct of a mucous gland was surrounded by a fairly broad mantle of deposited material Some of the most superficial striated muscle cells were also sur

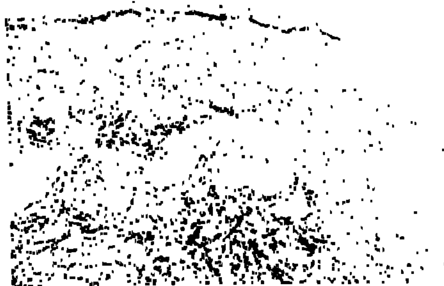


Fig 2 Patient VIII 1 Photomicrograph of the extirpated, immobilized right vocal cord Abundant deposits (palely stained) present To the left, indistinctly demarcated patches of deposits, forming a relatively superficial band To the right, the UWD deposits almost totally replace the normal tissues In the middle part of the figure a broad mantle of deposits surrounds an excretory duct of a mucous gland van Gieson elastin staining × 67

rounded by deposits In one location a group of such cells was included in material that was structurally suggestive of common UWD deposits, but which perhaps instead was connective-tissue hyalin, as it was stained as collagen with van Gieson's method, with the van Gieson elastin staining, with Herovici's method, with the PAS procedure and with Laidlaw's method In the connective tissue layer the relatively abundant elastic tissue was perhaps somewhat unevenly distributed and some elastic fibres showed signs of degeneration Furthermore, in some regions, vessel walls, apparently thickened due to UWD deposits, were more or less intensely stained as elastic tissue in van Gieson elastin-stained sections However, it usually was easy to identify elastin and deposits, respectively, with this staining method In sections stained with a red Sudan stain there were abundant amounts of sudanophil material in the subepithelial zone, but it was not possible to establish the exact relationship to the deposits

In the smaller biopsy specimen taken from the larynx more than 10 years previously changes similar to those in the extirpated vocal cord were seen In addition, a small area contained somewhat increased numbers of inflammatory cells

Patient VIII 2 Material, clinically found to be loosely fluttering in the glottis, was histopathologically found to consist of necrotic connective tissue containing abundant amounts of elastic tissue and many colonies of a fungus consisting of networks of fine hyphae A specimen of granulated mucous membrane and a specimen of cartilage like tissue below the vocal cords were found to consist of mucous membrane and degenerated cartilage respectively The surfaces of both specimens were partly covered by membranes containing abundant amounts of granulocytes In the mucous membrane specimen many areas contained large amounts of elastic tissue Near the surface some small blood vessels had somewhat thickened walls, seemingly due to UWD deposits, but unequivocal, more conspicuous deposits were not seen

Patient VIII 3 In the biopsy specimen from one vocal cord the vessel walls and connective tissue were almost totally replaced by deposits and only small areas of collagen could be seen The vessels had both relatively narrow and relatively wide lumina Only a few relatively small areas of elastin, showing degenerative changes, were present

Patient II In the biopsy specimen from the left vocal cord abundant deposits and irregularly distributed, seemingly degenerated elastic tissue were seen Affected blood vessels had both relatively wide and relatively narrow lumina A small subepithelial area was both slightly oedematous and contained increased numbers of inflammatory cells, *inter alia* plasma cells and granulocytes

With Herovici's method some thickened vessel walls assumed a reddish colour, and in some blood

TABLE 4 *Summary of Some Staining Reactions in UWD Deposits and Degenerated Elastin in Vocal Cord Specimens*

Specimen from	PAS			Reaction in degenerated elastic tissue		Reaction in deposits			
	vessel walls	extravascular deposits	degenerated elastin	van Gieson & Herovici	haematoxylin and eosin	alkaline Congo red	Thioflavin T	DMAB (tryptophan)	PTAH (fibrin)*
1	2	3	4	5	6	7	8	9	10
II (left)	+ +	+	(+)	+	/†	(+)	+	+/+ +″	–δ
II (right)	+ +	/	/	/	/†	–	/	/	/
IV	+ +	+ +	+	+ §	–†, $	–	+	+	–
VIII 1 (1957)	+ + +	+ +	+ +	/	/	–	+ +	+	–δ
VIII 1 (1968)	+ +	+/+ +	–	+	+	–	+ +	+ +″	–δ
VIII 3	+ +/+ + +	+ +	/	/§	/	–	+	+ +″	–δ

Explanations to the Table

In columns 5 and 6 the sign + indicates degenerated elastic tissue stained similarly as UWD deposits, in the other columns semiquantitative grading of the staining from + to + + + has been applied. The remaining symbols are used in a similar way as in Table 3

* Absence of colour reaction or a pale pink colour is designated as negative

§ A small area of elastic cartilage included in the specimen

† Some vessel walls, thickened due to UWD deposits, were fairly easy to observe as they assumed a fairly intense red colour

$ Degenerated elastin seemed to a large extent to be relatively unstained

″ Some vessel walls more intensely stained than the remaining tissues

δ Occasional clefts in the deposits assumed a dark colour in shades from blue to reddish violet

vessels different zones stained in pale rose, red and yellow could be identified, perhaps indicating deposits complex in composition

A biopsy specimen taken from the right vocal cord showed essentially the same histopathological changes There were not only deposits but also a large oedematous area

Patient IV A biopsy specimen from the right vocal cord showed abundant deposits and an irregular distribution of elastic tissue Vessels with relatively narrow as well as fairly wide lumina

types and a somewhat increased number of cells probably representing fibroblasts

Table 4 indicates some of the findings with the larger series of staining procedures It was found that degenerated elastin as well as the UWD deposits were stained yellowish by van Gieson's and Herovici s methods Furthermore, in some sections the degenerated elastic tissue was eosinophilic or showed some degree of PAS positivity and, consequently, was also difficult to differentiate from UWD deposits with these methods In some specimens the PAS positivity was most marked in vessel walls and in some specimens thickened vessel walls assumed a fairly intense red colour and consequently were easy to identify in haematoxylin and eosin stained sections

With the alkaline Congo red method a negative reaction was usually obtained and only in one specimen (II) was an exceedingly small patch in a vessel wall stained as amyloid Thioflavin T staining gave faint to moderate fluorescence in part of the UWD deposits, this was of lesser intensity than that seen in amyloid and was not sharply demarcated Other tissue constituents sometimes also displayed fluorescence, for instance in one specimen (VIII 1 1968) striated muscle cells showed the same degree of fluorescence as deposits In parallel sections not stained with Thioflavin T but otherwise treated in the same way fluorescence was also seen in most specimens although it was usually fainter than in the sections stained with Thioflavin T With the DMAB reaction for tryptophan the reaction in deposit varied from faint to moderate In those specimens in which the reaction was faint, at least in the specimen from IV, it should probably be considered as positive, as the concomitant reaction in striated muscle was extremely faint In some specimens the reaction for tryptophan was most marked in vessel walls

The UWD deposits were usually either unstained or assumed only a pale pink colour with the PTAH method Thus no fibrin was demonstrated Laidlaw's reticulin staining method indicated that the areas containing the most dense deposits lacked unequivocal collagen fibres and contained only a few reticular fibrils many of them in the shape of a spiral (VIII 1, VIII 3) In one specimen it was easy to observe that occasional thick fibre fragments in the deposits were stained as reticulin (VIII 1, 1968) Reticular fibrils could be seen in relation to some vessel walls (VIII 3, II, IV), in thickened vessel walls reticular fibrils could be absent, present only as a fine line surrounding the vessel wall or present in abundance as fine fibrils or lamellae in the wall

DISCUSSION

Past History

The early appearance of hoarseness in our patients is in accordance with previous reports about UWD (cf *Hofer* 1973) The present investigation shows that no unequivocal eliciting factor for the hoarseness can be obtained by a detailed anamnesis Many patients stated that the hoarseness becomes worse in damp weather Some of the earlier diagnoses show that physicians not familiar with the disease may mistake the laryngological findings for signs of more common diseases such as chronic laryngitis and singer's nodule Thus, the disease is easily overlooked and there is therefore reason to believe that it is more frequent than the number of 200 reported cases suggests

In agreement with *MacKinnon* (1968) and *Weidner et al* (1970) our studies show that the laryngeal changes at UWD may be demonstrated by tomography The vocal

cords are examined from different directions in tomography and laryngoscopy Therefore it would be of value if future studies included comparisons of the findings in these two types of examinations, performed almost simultaneously

Laryngoscopical Observations

To the review of patients reported up to 1972 (*Hofer* 1973), we are adding some data concerning laryngeal alterations Laryngeal changes have been described in most of the approximately 100 cases reported in the literature in which indirect or direct laryngoscopical examination was performed Changes in the true vocal cords have been described in about 75 cases, epiglottic changes in about 45, changes in the aryepiglottic folds in about 15 and changes in the false vocal cords in about 25 However, rarely are any of these structures directly stated to be normal This, of course, may reflect a tendency in brief case reports to confine descriptions of laryngeal changes to the true vocal cords. However, our findings indicate that changes in true vocal cords are more frequent than other laryngeal changes

The vocal cords are often reported to be thickened and to have irregular borders Also the Swedish patients studied laryngoscopically in the present examination had irregular borders of the vocal cords It seems therefore reasonable to assume that the wavy shape of the vocal cords prevents their perfect closure and thus causes the hoarseness The vocal cords may however be thickened but smooth as described by *Tompkins* & *Weinstein* (1954)

In most UWD patients the vocal cords have good motility From the findings in our patient VIII 1 it is however, apparent that in occasional patients the deposits may be abundant enough to cause immobilization of a vocal cord That the degree of hoarseness may be related to the degree of vocal cord changes visible at laryngoscopy, is indicated by the fact that the patient in our series with only slight hoarseness also had only insignificant laryngeal changes Unfortunately however, we have not had the opportunity to examine the patient without hoarseness Nor has it been possible to re examine any of those three patients described as using the false vocal cords In summary, the present study suggests that the hoarseness in UWD is usually due to air leakage between vocal cords with irregular borders

Histopathological Observations

The fact that the histopathological lesions in laryngeal biopsies have only been mentioned for about 10 cases in the literature (*Blodi et al* 1960 *Burnett* & *Marcy* 1963 *Cowan et al* 1961, *Fine et al* 1962, *Izaki et al* 1954, *Jensen* 1962, *McCusker* & *Caplan* 1962, *Thambiah et al* 1963, *Urbach* 1929) is not surprising, as the histopathological diagnosis of UWD can be established in biopsies from affected regions in the skin or oral mucosa, *i e* regions less sensitive than the vocal cords to the trauma of taking a biopsy specimen Only exceptionally has any detailed information been given concerning histopathological (*Cowan et al* 1961) or histochemical (*McCusker* & *Caplan* 1962) findings As far as comparison is possible, the reported findings in laryngeal biopsy specimens seem to resemble our observations However, it has not previously been reported that deposits may affect the subepithelial tissue in all its depth down to the musculature The presence of ordinary connective tissue hyalin around a few striated muscle cells (see histopathological description concerning case VIII 1) may either be a coincidental finding or indicate that UWD deposits can exceptionally be subject to an ageing process terminating at a stage at which they stain like collagen as has previously been described for amyloid and extravascular fibrin (cf *Lendrum et al* 1972) The significance of the oedema and the few inflammatory cells present in some specimens is for obvious reasons unclear

It is a well known fact that elastic tissue may show degenerative changes adjacent to deposits in UWD although it has apparently not been previously mentioned in relation to

vocal cord lesions Our findings should nevertheless be interpreted with caution, considering the normal abundance of elastic tissue in vocal cords and the fact that age related changes may also be present (cf *Ungar* 1967) In any case, it is apparent that only in van Gieson elastin stained sections is it possible to differentiate UWD deposits from elastin with a fair degree of certainty (see histopathological descriptions concerning case VIII 1)

Herovici's method was included in the present study because it gives more intense colours than van Gieson's method and because sections stained with Herovici's method do not tend to fade as rapidly as sections stained with van Gieson's method On the other hand, if interpretation is confined to non faded sections, van Gieson's method perhaps gives more consistent results on different occasions of staining with respect to the relation between the red and yellow colour shades

As in other locations, the UWD substance was PAS-positive, reacted in the DMAB method for tryptophan, was not stained as fibrin with the PTAH method, and did not give any clearly positive reaction in methods considered specific for amyloid Variations in intensity of the reaction for tryptophan from case to case may depend on the fact that usually tissues fixed in formalin have been examined (cf *Adams* 1957) In the present study reactions for amyloid could in principle be regarded as negative since hardly any amyloid was demonstrated with the alkaline Congo red method and the fluorescence seen in part of the deposits in some sections stained with Thioflavin T was less intense than in amyloid (cf *Cohen* 1967) One should also remember that some degree of fluorescence may be solely due to fixation in formalin (*Kawamura* 1969) It has only exceptionally been reported that part of the deposited material in UWD, either in the vocal cords or in other locations, gave positive staining for amyloid (cf *Hofer* 1973) It is impossible to know whether these few reports of amyloid in UWD deposits would be considered valid if current methods and current more rigorous criteria for the histochemical and histophysical identification of amyloid were applied The variable results regarding reticulin also agree with those reported from other locations Of course, these variations may raise the question whether the UWD deposits, like amyloid deposits (cf *Dreher & Vandre* 1972), may sometimes coat some reticular fibrils, making it impossible to demonstrate these fibrils with silver impregnation technique It was not possible to perform a detailed lipid histochemical study during the present investigation The occurrence of colonies of a fungus in necrotic material in one of our cases, and in a tracheostomy in a reported case (*Kindler* 1932) are probably only coincidental findings

The pathogenetic hypotheses concerning UWD have recently been reviewed (*Hofer* 1973) The observation that of all the various parts of the larynx, the vocal cords are particularly affected, is fully compatible with the hypothesis that lesions tend to appear especially in regions normally exposed to physiologic traumata Interpretation of the fact that anamnesis in some patients indicates the presence of hoarseness since birth must, at our present stage of knowledge, remain open

The pathological findings in the present study are compatible with the suggestion that deposits in UWD first appear in the walls of small blood vessels It is however, still uncertain whether the disease actually is, as *Laymon & Hill* (1957) suggest, characterized by "a congenital irritability or fragility of the terminal vessels, with transudation of lipids and proteins into the tissues"

Supported by grants from the *Medical Faculty in Umeå*, the *Swedish Diabetes Association*, the *Swedish Society for Medical Research*, and the *Edvard Welander Foundation*

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LOCALIZATION OF IMMUNOGLOBULINS IN URINARY BLADDER TUMOURS

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Biopsy specimens from primary tumours of the urinary bladder in 36 patients were examined for the presence of immunoglobulins, using a direct immunofluorescence technique The tumours were also studied by normal histological methods to determine the malignancy grade and degree of invasive growth as well as the presence of lymphocytes and plasma cells An immunoglobulin estimation in urine and serum from the patients was performed by immunodiffusion The IgM demonstrated in lymphocytes and plasma cells of the tumours connective tissue stroma was found to be directly related to the malignancy grading There was even a correlation to the urinary output of IgM, but not to its serum concentration These results further support the view that an immune reaction occurs in epithelial tumours of the urinary bladder, and that an estimation of the urinary output of IgM can be of diagnostic value in these cases

Patients with transitional cell tumours show a high urinary excretion of immunoglobulins, particularly of IgM, and a low excretion of α-1-globulin and transferrin compared with patients with proteinuria due to glomerulonephritis and pyelonephritis (*Johansson et al* 1971) Moreover, the amount of IgM in the urine is directly related to the size of the tumour, as judged from a cystoscopical examination (*Johansson & Kistner*) It therefore appears that IgM passes into the urine from the tumour, but the actual presence of IgM in these tumours has never been demonstrated In the present study, the presence of IgM in bladder tumours is investigated by immunofluorescence and the concentration of IgM in serum and urine is examined by immunodiffusion

Received 21 iii 74 Accepted 21 iii 74

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MATERIAL AND METHODS

Investigations were performed on 36 consecutive patients who were admitted to the department of Urology on account of a primary tumour of the urinary bladder Although most of these patients had microscopical haematuria, patients with gross macroscopical haematuria were not included in the study Samples of urine and serum as well as a tumour biopsy from each patients were examined

Serum creatinine was less than 1 3 mg/100 ml in 27 patients, 1 3-1 5 mg/100 ml in 5 and 1 5-1 8 mg/100 ml in the remaining 4 Twenty eight of the patients had negative urine cultures, growth of enterococci was seen in 5, of E coli in 2, and of staphylococcus albus in 1 Patients with positive urine cultures, however, showed no other signs of urinary tract infection In all patients, the serum concentration and the 24 hours urinary excretion of immunoglobulins was determined (*Johansson & Kistner*)

Biopsy specimens from the tumour and from the intact bladder wall as far removed from the tumour as possible were taken during a cytoscopical examination Cryostat sections were prepared, for immunofluorescence reactions, from the biopsies, after which the frozen blocks were fixed in a 10 per cent neutral solution of formalin and embedded in par

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Acta path microbiol scand Sect A, 82, 559-563, 1974

LOCALIZATION OF IMMUNOGLOBULINS IN URINARY BLADDER TUMOURS

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Biopsy specimens from primary tumours of the urinary bladder in 36 patients were examined for the presence of immunoglobulins, using a direct immunofluorescence technique The tumours were also studied by normal histological methods to determine the malignancy grade and degree of invasive growth as well as the presence of lymphocytes and plasma cells An immunoglobulin estimation in urine and serum from the patients was performed by immunodiffusion The IgM demonstrated in lymphocytes and plasma cells of the tumours connective tissue stroma was found to be directly related to the malignancy grading There was even a correlation to the urinary output of IgM, but not to its serum concentration These results further support the view that an immune reaction occurs in epithelial tumours of the urinary bladder, and that an estimation of the urinary output of IgM can be of diagnostic value in these cases

Patients with transitional cell tumours show a high urinary excretion of immunoglobulins, particularly of IgM, and a low excretion of α 1-globulin and transferrin compared with patients with proteinuria due to glomerulonephritis and pyelonephritis (*Johansson et al* 1971) Moreover, the amount of IgM in the urine is directly related to the size of the tumour, as judged from a cystoscopical examination (*Johansson & Kistner*) It therefore appears that IgM passes into the urine from the tumour, but the actual presence of IgM in these tumours has never been demonstrated In the present study, the presence of IgM in bladder tumours is investigated by immunofluorescence and the concentration of IgM in serum and urine is examined by immunodiffusion

Received 21 iii 74 Accepted 21 iii 74

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MATERIAL AND METHODS

Investigations were performed on 36 consecutive patients who were admitted to the department of Urology on account of a primary tumour of the urinary bladder Although most of these patients had microscopical haematuria, patients with gross macroscopical haematuria were not included in the study Samples of urine and serum as well as a tumour biopsy from each patients were examined

Serum creatinine was less than 1.3 mg/100 ml in 27 patients, 1 3 1 5 mg/100 ml in 5 and 1 5 1 8 mg/100 ml in the remaining 4 Twenty eight of the patients had negative urine cultures, growth of enterococci was seen in 5, of E coli in 2, and of staphylococcus albus in 1 Patients with positive urine cultures, however, showed no other signs of urinary tract infection In all patients, the serum concentration and the 24 hours' urinary excretion of immunoglobulins was determined (*Johansson & Kistner*)

Biopsy specimens from the tumour and from the intact bladder wall as far removed from the tumour as possible were taken during a cytoscopical examination Cryostat sections were prepared, for immunofluorescence reactions, from the biopsies, after which the frozen blocks were fixed in a 10 per cent neutral solution of formalin and embedded in par

affin for the production of routine histological sections

Immunofluorescence

The cryostat sections for direct immunofluorescence were prepared as follows

IgM As regards the entire series of 36 patients, three sections were cut from each block and incubated with fluorescein conjugated sheep-anti human IgM serum (SBI, Stockholm)

IgG As regards 30 out of the 36 patients, three sections were cut from each block and incubated with fluorescein conjugated sheep anti human IgG serum (SBI, Stockholm)

IgA As regards 10 out of the 36 patients, three sections were cut from each block and incubated with fluorescein conjugated sheep anti human IgA serum (SBI, Stockholm)

The reaction with fluorescein conjugated anti immunoglobulin serum was inhibited in three sections from each block by pretreatment with the corresponding non conjugated anti immunoglobulin serum

All sections were studied by fluorescence microscopy with UV light

Histology

The histological sections were stained with haematoxylin-eosin and van Gieson's connective tissue stain Particular attention was paid to the malignancy of the tumour as judged from its cellular characteristics (*Vergkvist et al* 1965), its degree of invasion, and the infiltration of lymphocytes and plasma cells in the stroma and bladder wall Only slight cellular infiltration was denoted +, a moderate amount ++ and a massive infiltration +++

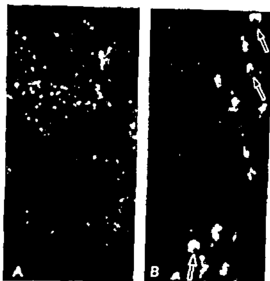


Fig 1 A Fluorescence microphotograph of section from urinary bladder tumour incubated with fluorescein conjugated anti human IgM serum Groups of fluorescent cells are seen and these are located in the connective tissue stroma Non fluorescent areas correspond to tumour cells × 100

B Fluorescence microphotograph of section from urinary bladder tumour incubated with fluorescein conjugated anti human IgM serum In many fluorescent cells dark areas (= nuclei) are excentrically located (arrows) Left and bottom right, non fluorescent tumour cell areas × 300

RESULTS

Immunofluorescence

IgM Test sections from 15 of the 36 tumours showed positive immunofluorescence reaction for IgM whereas all control sections were negative, as were the sections from the non tumourous bladder wall Positive reaction were judged by the occurrence of intensely fluorescent cells in the connective tissue stroma of the tumour (Fig 1 a) Most of these were classified as plasma cells because of the eccentric location of their nuclei (Fig 1 b), but some others were probably lymphocytes The tumour cells were not fluorescent

IgG A positive reaction was observed in 6 of the 30 tumours examined, whereas the control sections were negative with the exception of one bladder wall biopsy Fluorescence was again located in plasma cells and lymphocytes, but in three cases even in a more diffuse fashion in the stroma (Fig 2) The tumour cells were not fluorescent

IgA A positive reaction was observed in 2 of the 10 tumours examined whereas all the control sections, including those from the non tumourous bladder wall were negative Fluorescence was located in cells in the connective tissue stroma but not in the tumour cells Diffuse fluorescence in the stroma was absent

Both patients who were positive for IgA were also positive for IgG and IgM Among the remaining 13 patients who were positive for IgM, only one showed a positive reaction for IgG, whereas the remaining 3 patients with a positive reaction for IgG were negative for IgM

nective tissue stroma of the tumour in 17 cases, in 6 of these, it could also be seen, though to a lesser extent, in the biopsy from the distant non-tumourous bladder wall Twelve cases showed invasion of the tumour into the bladder wall

The biopsy specimens from non-tumourous bladder wall showed normal histological pictures except for a sparse distribution of lymphocytes and plasma cells in the lamina propria of some specimens One of these showed a positive immunofluorescence reaction for IgG

The immunofluorescence reaction, cellular infiltration and degree of invasion were related to the various grades of malignancy (Table 1) A positive reaction for IgM was most commonly seen in the more malignant tumours and this relationship was significant for grade M III The biopsies from the 2 M IV cases in which the IgM reaction was negative were almost devoid of stroma and consisted mainly of fragments of a highly polymorphous tumour



Fig 2 Fluorescence microphotograph of section from urinary bladder tumour incubated with fluorescein conjugated anti human IgG serum A strand of *diffuse stromal fluorescence is seen Above and* below, non fluorescent tumour cell areas × 140

A clear correlation between a positive IgM reaction and stromal infiltration of lymphocytes and plasma cells could be demonstrated Grade M I tumours showed an infiltration of cells only in one instance, the solitary IgM positive case in the group Grade M II tumours included 3 IgM positive cases, of which 2 showed either moderate or massive cellular infiltration whereas the biopsy material from the third case was of poor quality with a very sparse stromal component Four M II tumour were negative for IgM but

Histology

All cases showed transitional cell tumours which were classified according to *Bergkvist et al* (1965) as malignancy grade I in 3 cases, grade II in 19, grade III in 10 and grade IV in 4 There was a significant infiltration of lymphocytes and plasma cells in the con

TABLE 1 *The Relationship between Positive IgM Fluorescence Reaction, the Infiltration of Lymphocytes and Plasma Cells the Degree of Bladder Wall Invasion and the Grade of Malignancy in 36 Tumours of the Urinary Bladder*

Grade	No	IgM reaction	Cell infiltration			Invasion		
			+	+ +	+ + +	l p	musc	p v
M I	3	1		1			–	
M II	19	3	4	1	1		1	2
M III	10	9	4	4		7	1	–
M IV	4	2	2			–		–

l p = lamina propria musc = muscle wall, p v = perivesical tissue

TABLE 2 *Serum Concentration and Urinary Excretion of IgM in 36 Patients with Tumours of the Urinary Bladder Related to Their Immunofluorescence Reaction of IgM*

IgM reaction	No	Serum IgM mg/100 ml	Urine IgM mg/24 hours
Positive	15	81 ± 66	7 4 ± 2 2
Negative	21	84 ± 35	2 4 ± 0 9

Mean values ± SE are given The difference in urinary IgM in the two groups is significant ($p < 0\ 001$)

showed a slight cellular infiltration As regards grade M III tumours 9 IgM positive cases were seen and all but one of these showed slight to moderate cellular infiltration the exception being of poor histological quality and almost devoid of stroma No cellular infiltration was seen in the IgM negative case Both cases of M IV tumours in which IgM was demonstrated showed a cellular infiltration in the stroma but as mentioned above the biopsies from the other two tumours contained hardly any stroma

The degree of invasion of the tumours did not correlate to the IgM reactions but tended to be inversely related to cellular infiltration Thus all three M II tumours in which invasion of the bladder wall was demonstrated were devoid of cellular infiltration and negative for IgM

In Table 2 the serum concentration and urinary excretion of IgM is related to immunofluorescence in the tumour biopsy It can be seen that patients with positive immunofluorescence excrete significantly more IgM in the urine than patients with negative immunofluorescence ($p < 0\ 001$) whereas the serum concentrations do not differ significantly

DISCUSSION

It has been shown that the urine from patients with transitional cell tumours of the urinary tract contains varying amounts of IgM (*Johansson et al* 1971) which appears to be directly related to the area of mucosa involved in the tumour growth (*Johansson & Kistner*) In the present study, IgM was demonstrated in several of the tumours more over the urine from these patients contained significantly more IgM than that from patients with 'IgM negative tumours thus indicating that the production of IgM was dependent on the tumour itself It therefore follows that urinary analysis for IgM could *be useful in the diagnosis and follow up of* patients with transitional cell tumours of the urinary tract

Those cases in which an increase of urinary IgM was paradoxically seen in an IgM negative tumour may have been a result of an inadequate biopsy Large tumours may well produce large amounts of IgM although a small biopsy specimen appears IgM negative or shows only a sparse distribution of the immunoglobulin Conversely small tumours may appear rich in IgM on biopsy but still produce only small amounts of the immunoglobulin in the urine The mechanism by which IgM is transferred from the tumour to the urine however remains obscure

The hypothesis that stromal infiltration of cells containing immunoglobulins reflects a specific immune reaction evoked by tumour *antigens is born out by the present study* and corroborated by previous studies of patients with carcinoma of the urinary bladder (*Bubenik et al* 1970 and *Bubenik et al* 1970) That these immunoglobulins in the stroma are secondary non specific effects such as simultaneous infection (*Uehling et al* 1968) cannot totally be excluded The extracellular distribution of IgG seen in some of the tumours is suggestive of such a non specific reaction In some cases the degree of cellular infiltration observed in biopsy specimens from even non tumourous parts of the bladder wall suggested an immune reaction but the small size of the biopsy and scanty distribution of immunoglobulins may explain the absence of a positive immunofluorescence reaction

The present study primarily demonstrated the presence of IgM in plasma cells and lymphocytes in the stroma of urinary bladder

tumours, with a concurrent increase of IgM excretion in the urine These observations cannot evaluate the effect of an immunological reaction as a limiting factor in tumour growth, nor can they ascertain whether the immune reaction appears early or only when the tumour becomes increasingly aggressive Whereas *Sarma* (1970) found a heavy cellular infiltration to be a good prognostic sign, others deny this correlation (*Tanaka et al* 1970) The absence of cellular infiltration and IgM in three M II tumours which showed marked invasive tendencies, suggests that an immunological reaction may be of some importance in limiting tumour growth On the other hand, the remaining nine M II tumours also were devoid of cellular infiltration and did not display any invasion An analysis of the more malignant tumours (M III and M IV) does not elucidate this problem any further, the only constant feature being a stromal infiltration of IgM positive cells, quite independent of the invasive characteristics of the tumour

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This study was supported by the *Swedish Cancer Society* (Project No 523-71-01X)

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Acta path microbiol scand Sect A, 82, 564-570, 1974

ENHANCED DEVELOPMENT OF RETICULUM CELL NEOPLASMS FOLLOWING SUBCUTANEOUS INOCULATIONS OF CELL-FREE FILTRATE OF *EHRLICH'S* ASCITES CARCINOMA

A Study in Adult, Female Mice

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Adult female mice from a random bred, closed colony were inoculated subcutaneously with vital cells from *Ehrlich's* ascites carcinoma or with a cell free filtrate from EAC. After both types of treatment, the mice revealed a high incidence of reticulum cell neoplasms type B. It is suggested that an oncogenic virus may be implicated in the RCN development.

Preliminary studies at this Institute have shown a high incidence of reticulum cell neoplasms (RCN) in adult mice surviving immunization with vital cells of *Ehrlich's* ascites carcinoma (EAC) (*Myking* 1969). The development of lymphomas in mice has also been reported following the injection of cell free filtrate of transplantable mouse tumours (*Graffi et al* 1956, *Friend* 1957, *Moloney* 1960, *Stansley* & *Soule* 1962). This study was carried out to confirm the previous observation with the vital EAC, and to investigate the possible lymphoma inducing activity of a cell free filtrate of this tumour.

EAC tumour cells have been shown to evoke immunological reactions (*Thunold* 1967 and 1968). The ascites fluid contains allogenic proteins and inflammatory cells which also may expose the recipients to immunological stimulation. Immunological stimulation has been suggested as a cause in the development of reticulosarcomas in mice (*Metcalf* 1961, *Tyndall et al* 1972), and a separate study has therefore been set up to investigate this problem (to be published).

Received 11 i 74 Accepted 12 iii 74

MATERIAL AND METHODS

The *mice* used were of the closed colony kept at this Institute (*Hartveit* 1961). They were kept in cages containing up to 20 animals, with sawdust as bedding, food (commercial pellets) and water ad libitum.

The *tumour* was the EAC that has been kept at this Institute by repeated intraperitoneal transplantations for many years (*Hartveit* 1961). Tumour ascites from several 5 and 9 day transplants was pooled and used for injection at a dosage of 0.05 ml within 2 hours of collection. The tumour cell counts in two pools used for vital cell injection and preparation of cell free filtrates were 12 × $10^4/mm^3$ and 11 × $10^4/mm^3$, respectively. In the third tumour pool used only for preparation of cell free filtrate the final cell count was 11 × $10^4/mm^3$.

Cell free tumour filtrate was prepared by freezing the EAC cell suspension twice to −70° C for fifteen minutes, thawing to 37° C and centrifuging at 23 500 *g* for 20 minutes at 4° C. The super

natants were diluted 1:5 in buffered physiological saline (pH 7.2) and passed through a 0.45 μ millipore filter. The filtrates were kept cool on melting ice during filtration and injections. All injections were completed within 3 hours of collection.

Statistics. The P values have been estimated according to the chi square test.

Experiment 1

Lymphomas following injection of vital EAC cells. One hundred and five female mice, aged 19.4 ± 2.2 weeks (Mean ± SD) were immunized with vital EAC cells as recommended by *Andervont* (1932). Tumour ascites was injected subcutaneously into the tails which were amputated under ether anaesthesia near the roots after 9 days when the tumour growth was established. Six days thereafter a challenge dose of 5 × 10^6 tumour cells was given subcutaneously onto the backs. Mice developing local EAC tumours on the backs or at the tail roots or developing EAC metastases, were excluded from the experiment. The final *experimental group A* in which the subsequent development of lymphomas could be studied, consisted of 36 mice that survived treatment without recurrence of the EAC.

A further 112 female mice, aged 17.6 ± 6.2 weeks received only one injection of EAC into the tails, and no challenge dose after tail amputation. Of these 46 survived treatment and formed *experimental group B.*

Experiment 2

Lymphomas following injections of cell free filtrate of EAC. Thirty three female mice, aged 27 ± 0 weeks were given 3 injections of 0.25 ml cell free EAC filtrate subcutaneously on their backs at weekly intervals. Twenty three female mice of similar age (27.6 ± 0.6 weeks) were given corresponding saline injections and served as controls. One of these mice was destroyed by cannibalism and therefore excluded.

The mice in both experiments were all kept until they died or were killed when clearly moribund. The age at death was recorded.

They were examined for the presence of tumours in the lymphoreticular system and in other organs. The spleen, liver and kidneys from all the mice were examined histologically, as were other organs suspected of lymphoreticular malignancies or other kinds of tumours. The tissue was fixed in 4 per cent buffered formalin, paraffin embedded and stained with haematoxylin and eosin and/or Giemsa stain for histological typing and confirmation of the diagnosis.

Diagnosis and Classification of Lymphomas

The material available for microscopical examination was for the most part tissue from animals dying spontaneously, hence blood smears were not made routinely. Classification was thus based on the histological and macroscopical picture.

Tumours arising from cells in the reticulo-endothelial system and myeloid tissue were classified as RCN, lymphosarcomas or granulocytic neoplasms. The histological criteria were those described by *Dunn* (1954) and *Dunn & Deringer* (1968).

TABLE 1. *Experiment 1. Number of Mice Developing Lymphomas Following Injection of Vital EAC Cells*

Treatment	Group A Vital EAC twice	Group B Vital EAC once
RCN type B	19/36 (53%)	22/46 (48%)
Lymphosarcomas	2/36 (5%)	1/46 (2%)
Granulocytic neoplasms	0	1/46 (2%)

RESULTS

Lymphoma Development Following Inoculation of Vital EAC Cells (Experiment 1, Table 1)

The incidence of histologically confirmed RCN was high in both groups, 53 per cent and 48 per cent for groups A and B, respectively. This difference between mice receiving two (A) and one (B) injections of EAC is not statistically significant. In addition two lymphosarcomas were recorded in group A and one lymphosarcoma and one granulocytic neoplasm in group B.

Lymphoma Development Following Inoculation of EAC Filtrate (Experiment 2, Table 2)

The incidence of RCN following injection of EAC filtrate was 58 per cent compared to 24 per cent in the control group. This difference is statistically significant ($P < 0.008$). One lymphosarcoma was seen in each of these groups.

The age at death in both experiments related to the presence or abscence of lymphoma development is given in Table 3. The mean life span in the different groups varied from 84 to 108 weeks. However, the dif-

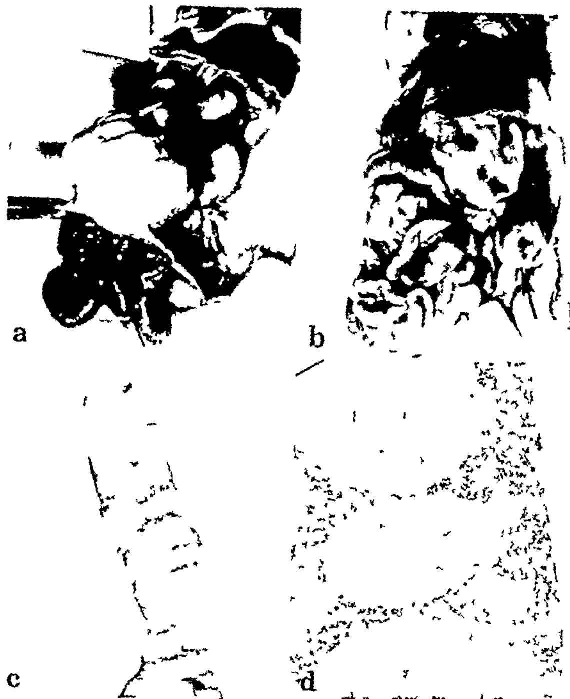


Fig 1 Type B reticulum cell neoplasms a) *Gross* enlargement of mesenteric lymph node The arrow points to the lower border of a moderately enlarged spleen (Spleen weight [illegible]20 mg) b) Greatly enlarged mesenteric lymph node (below the edge of the liver) and spleen (arrow pointing at the lower border) Spleen weight 1257 mg c Macroscopical picture of a section from an enlarged spleen The splenic nodules are enlarged and partly confluent d) Histological section *from* the same specimen as c Giemsa stain × 50

TABLE 2 *Experiment 2 Number of Mice Developing Lymphomas Following Injection of Cell Free EAC Filtrate Compared to Saline Treated Controls*

Treatment	EAC filtrate	Saline
RCN type B	19/33 (58%)	5/21 (24%)
Lymphosarcomas	1/33 (3%)	1/21 (5%)
Granulocytic neoplasms	0	0

TABLE 3 *Life Span in Weeks of Mice with and without Lymphomas Mean ± SD*

	Group A EAC twice	Group B EAC once
Experiment 1		
Lymphomatous mice	84±18 5	92±18 4
Non lymphomatous mice	88±19 7	105±14 5
	EAC filtrate	Saline
Experiment 2		
Lymphomatous mice	88 0±20 7	108 ± 8 3
Non lymphomatous mice	99 2±21	97 6±20 7

ferences between lymphomatous and non-lymphomatous mice in each experiment, were statistically not significant

The distribution of the lesions in mice with reticulum cell neoplasms was similar in both experiments and with no difference between control mice and treated mice Either the spleen or the mesenteric lymph nodes, or both, were enlarged, often to a marked degree (Fig 1 a and 1 b) In the spleen, the malignant lymphoreticular proliferation characteristically involved the white splenic pulp, leaving the red pulp uninvolved (Fig 1 c and 1 d) Often the liver, the retroperitoneal lymph nodes and the *Peyer*'s patches were involved, in some cases also the peripheral lymph nodes In most cases, the thymus was atrophic, but was involved in a few instances where extensive tumour spread in other lymphoid organs was present as well

Types of Lymphomas

In both experiments, the histological character of nearly all the lymphomas was consistent with the type B reticulum cell neoplasms of *Dunn* The most frequent finding was a mixture of neoplastic reticulum cells and lymphocytes (Fig 2 a) A few granulocytes and plasma cells could usually be found In some specimens, tumour giant cells not unlike the *Reed-Sternberg* giant cell were present (Fig 2 b) as well as varying degrees of fibrosis (Fig 2 c) Although this was the usual histological picture, variation along two main lines was seen At the one extreme, lymphocytic predominance was marked (Fig 2 a, 3 a and 3 b) at the other lesions similar to *Hodgkin*'s sarcoma or reticulosarcoma in man occurred with almost complete lymphocytic depletion (Fig 2 d) A characteristic feature in some of the lymphomas was a typical starry-sky appearance (Fig 3 a and 3 b) Classification was, however, made without regard to this pattern

DISCUSSION

The present work shows a high incidence of RCN in mice surviving inoculation with vital EAC cells This is in keeping with the preliminary results which formed the basis for this study and was not dependent on whether one or two injections were given

Inoculation with a cell free filtrate of this tumour gave a similar result While 24 per cent of control mice treated with saline developed RCN the incidence was 58 per cent in mice given EAC filtrate

The mice with RCN died at an average age of 84–108 weeks This agrees with previous reports on type B RCN (*Dunn & Deringer* 1968) The finding that the life span of animals developing RCN was not shortened, was unexpected, but in keeping with experience from chronic lymphocytic leukaemia in AKR mice (*Arnesen* 1956) Similar records on RCN [illegible]

typ [illegible]

ported findings Thymic involvement has pre-

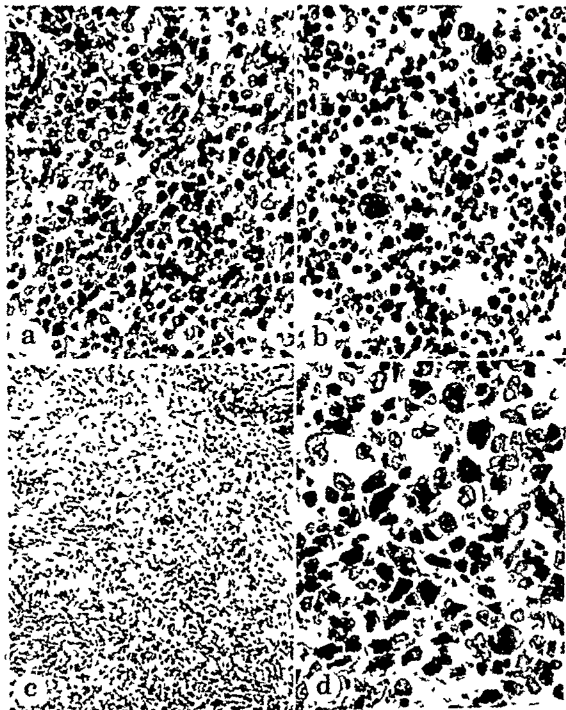


Fig 2 Different h stol g cal patterns of type B neoplasms a) Spleen Mixture of neoplastic reticulum cells and lymphocytes The most frequently recorded histological picture H + E × 350 b) The same basic histological pattern as Fig 2a but with tumour giant cells similar to *Reed-Sternberg* cells S ch giant cells were seldo recorded H + E × 350 Thymus c) Fibrosis at the border of enlarged splen c nodule H + E × 150 d Pleomorphic picture from spleen with large atypical reticulum cells but almost depl ted of lymphocyte Lesions s milar to this were sometimes difficult to separate from a pleon orphic type A neoplasm Th pattern was frequently recorded in this series and resembles a pleomorphic reticulosarcoma or a *Hodgk n s* sarcoma in man H + E × 600

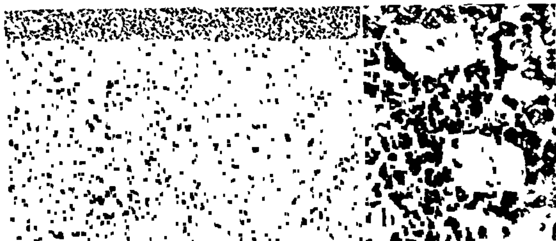


Fig 3 Sections from mesenterial lymph node showing a typical 'starry sky' appearance The cells are lymphocytes of a lymphoblastic type, showing a more pleomorphic picture than well defined lymphosarcomas The lesion was classified as a type B neoplasm due to the histological picture in the spleen which is shown in Fig 2a a) H+E × 150 b) H+E × 600

viously been described only in wild mice (*Dunn & Deringer* 1968)

A significant increase in the incidence of type B RCN followed the injection of sub cellular material obtained from our EAC line into *adult mice* of a random bred colony RCN induction by subcellular material has previously only been reported to occur in *suckling mice* of certain inbred strains One report described the development of RCN type B after inoculation with subcellular material from EAC in newborn BALB/c mice (*Stansley & Soule* 1962) These tumours were successfully propagated with cell free extracts Development of RCN has been demonstrated in thymectomized BALB/c mice inoculated with a cell free preparation obtained from lymphocytic neoplasms induced in the same strain by the *Moloney* agent (*Dunn & Deringer* 1968) RCN has also been induced in BALB/c mice with cell free preparations obtained from spontaneous RCN in the SJL/J strain (*Fujinaga et al* 1970), virus particles identical to murine leukaemia virus type C being observed in the spontaneous as well as in the induced RCN

The present study does not demonstrate the nature of the active agent or its mode of action although several theoretical possibilities exist In view of previous experiments on mice lymphomas in general, and the studies on type B RCN quoted above, the possibility that an oncogenic virus may be implicated is most attractive

This work has been carried out with financial support from the *Norwegian Cancer Society*

Dr *Thelma B Dunn* at the National Cancer Institute, Bethesda, Maryland has reviewed some of the histological specimens, for which I am very grateful I am most grateful to Professor *Flora Hartveit* for valuable advice and criticism, and to Professor *Gunnar Haukenes,* the cell free preparations being prepared in his department The skilled technical assistance of Mrs *Anne Marie Sundstrom* is gratefully acknowledged

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Acta path microbiol scand Sect A, 82, 571–577, 1974

PARTICLES SIMILAR TO MOUSE LEUKAEMIA VIRUS IN *EHRLICH*'S ASCITES CARCINOMA AND IN RETICULUM CELL NEOPLASMS TYPE B

An Electron Microscopic Study in Mice

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Particles with morphological features similar to mouse leukaemia virus have been detected in a transplant of *Ehrlich*'s ascites carcinoma (EAC) and in two reticulum cell neoplasms (RCN) of histological type B One RCN arose in a mouse subsequent to inoculation with vital EAC cells, the other, in a sham treated mouse The virus is considered a possible agent causing enhanced development of reticulum cell neoplasms

Several reports on the induction of leukaemias and lymphomas in mice after injection of cell free filtrates from transplantable mouse carcinomas and sarcomas are available (*Graffi et al* 1956, *Friend* 1957, *Moloney* 1960, *Stansley* & *Soule* 1962) Well-established lines of murine leukaemia virus (MLV) have been recovered from some of these induced lymphomas

Enhanced development of reticulum cell neoplasms (RCN) type B have been recorded following the inoculation of cell free filtrates or vital cells of *Ehrlich*'s ascites carcinoma (EAC) into adult female mice (*Myking* 1974) The present study was carried out to investigate whether virus particles were present in a transplant of our EAC line and in reticulum cell neoplasms in the reported series

Received 11 i 74 Accepted 12 iii 74

MATERIAL AND METHODS

Tissues from a solid subcutaneous 6 day old EAC transplant and from mesenteric lymph nodes and the spleens of two mice with reticulum cell neoplasms (RCN), histological type B, were examined The animals and tumours dealt with in this study are described elsewhere (*Myking* 1974)

The EAC line used has been kept at the Gade Institute for many years (*Hartveit* 1961) One of the type B neoplasms occurred in a female mouse 13 months after inoculation with vital EAC cells The other neoplasm originated in a sham treated control mouse At the age 15 and 16 months, respectively, the mice were in a moribund condition The diagnostic criteria used in macroscopical and histological characterization of the tumours were those of *Dunn* (1954) and *Dunn* & *Deringer* (1968) (Fig 1 a–b)

The mice were killed by cervical dislocation, the tissues were dissected free and transferred to physiological saline The tissues were then rapidly sliced and immersed into a chilled 2 5 per cent glutaraldehyde solution in isotonic glucose phosphate buffer, pH 7 4 (*Millonig* 1962), for one hours prefixation After a brief rinse in the buffer, the tissues were post fixed for one hour in *Millonig*s 1 per cent osmiumtetroxide, then again

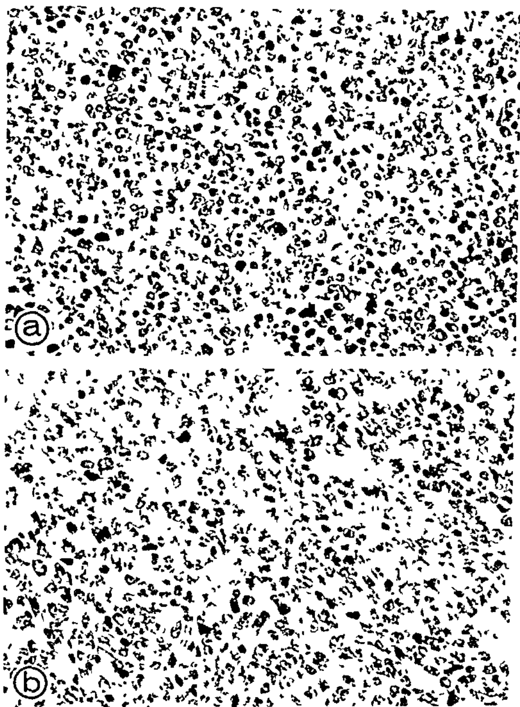


Fig 1 a) From the spleen Mixed pattern of neoplastic reticulum cells and smaller lymphocytic cells characteristic of reticulum cell neoplasms of histological type B The tumour arose subsequent to inoculation with vital EAC cells Haematoxylin eosin × 350 b) Similar tumour picture as in a) From the spleen of a sham treated mouse Haematoxylin eosin, × 350

rinsed in buffer, dehydrated through an ascending series of ethanol, and embedded in Epon 812 Ultrathin sections were cut with glass knives on an LKB ultramicrotome, mounted on formvar and carbon coated single hole copper grids, stained with lead citrate (*Reynolds* 1963), and examined in a *Philips* EM 300 electron microscope

RESULTS

Virus-like particles were found in all examined specimens They were seen in most cells in the EAC tumour, and also in the reticulum cell neoplasms, situated extracellularly, within the cytoplasmic matrix, in cytoplasmic vacuoles, and within cisternae of the rough endoplasmic reticulum None were observed within the cell nuclei

We have compared the morphology of various particles found in the EAC with corresponding particles seen in the RCN tumours Type A particles with a diameter 80 100 mμ were in both cases found in the cisternae (Fig 2 a–b), in the cytoplasmic matrix (Fig 3 a–b), and in extracellular spaces (Fig 5 a b) Type C particles measuring 100–140 mμ were found in the extracellular spaces (Fig 6 a b), in some specimens C particles were seen within cytoplasmic vacuoles (Fig 7 a–b) Doughnut shaped A particles were found budding from the cell surface (Fig 4a b), more rarely protruding into cisternae of the endoplasmic reticulum No type B particles characteristic of the murine mammary carcinoma (*de Harven* 1968) were observed

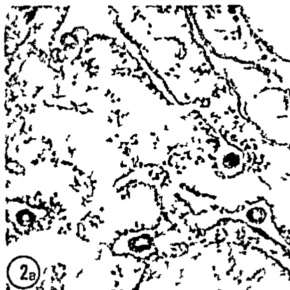
Concerning the distribution of particles in the examined tumours, some differences were recognized In the EAC tumour, intracisternal A particles were more numerous than in the reticulum cell neoplasms On the other hand, C particles lying freely within cytoplasmic vacuoles or in the extracellular spaces were more abundant in the reticulum cell neoplasms The occurrence of budding A particles was recorded more frequently in the reticulum cell neoplasms than in the EAC tumour

DISCUSSION

The particles found in the EAC transplant and in the two examined reticulum cell neoplasms have morphological characters corresponding to murine leukaemia virus (*Dalton et al* 1966, *de Harven* 1968) In a series of RCN of the histological type A, absence of virus particles was reported (*Chino et al* 1971) *Fujinaga et al* (1970) examining RCN of the histological types A and B, induced with cell free extracts from spontaneously occurring RCN in the SJL/J strain of mice, reported C particles, budding particles and intracisternal A particles in RCN of both histological types A and B Their findings correspond closely to the results recorded from the two reticulum cell neoplasms examined in our material

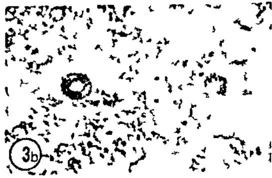
In the EAC, virus particles with morphological characters of A type particles, have previously been described by *Friedländer & Moore* (1956), *Adams & Prince* (1957), *Wessel & Bernard* (1957), *Yasuzumi & Sugihara* (1958), and *Wolf* (1959) However, *C type particles* in the EAC do not seem to have been reported previously Many investigators consider that C-type particles are involved in the leukaemogenic process and are observed in all virus induced murine leukaemias (*Bernard & Gross* 1959, *Dalton et al* 1961, *Bernard & Grandboland* 1962)

The EAC used contained lymphocytes, macrophages and red cells besides the tumour cells Being harvested from random bred mice the EAC, and the crude extracts made from this tumour will contain material antigenic to the mice into which it is inoculated Crude extracts from allogenic lymphocytes have been reported to induce chromosomal aberration which has been suggested as a step in the genesis of malignancy (*Fialkow* 1967, *Fialkow et al* 1968) An other theory to be considered is the possible effect of antigenic stimulation (*Metcalf* 1961, *Schwartz & Beldotti* 1965, *Cornelius* 1972) Lymphoma induction in mice has also been reported after injection of RNA isolated from EAC (*Lacour et al* 1960, *Bressler et al* 1962) Thus, while

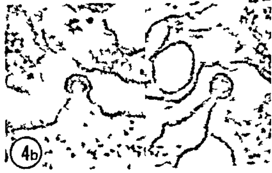

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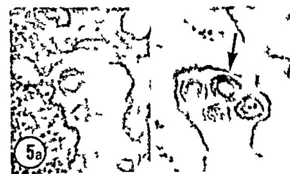

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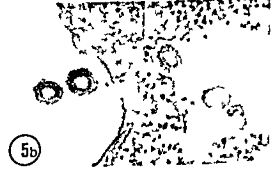

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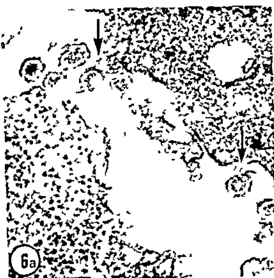

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4b


5a


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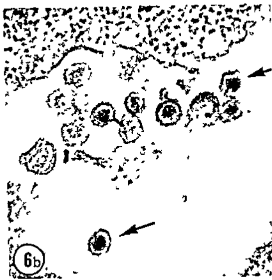






Fig 6 a) EAC C particles (arrows) in an extracellular space × 68200 b) Reticulum cell neoplasm type B C particles (arrows) in an extracellular space × 68200

Fig 7 a) EAC C particles (arrows) localized within cytoplasmic vacuoles × 68200 b) Reticulum cell neoplasm type B C particles (arrows) in phagocytic vacuoles of a macrophage × 68200

Fig 2 a) EAC Intracisternal A particles × 41800 b) Reticulum cell neoplasm type B Intracisternal A particles × 41800

Fig 3 a) EAC Naked intracytoplasmic A particles × 41800 b) Reticulum cell neoplasm type B Naked intracytoplasmic A particles. ×41800

Fig 4 a) EAC Budding A particles, into a cisterna (left) from the cell surface (right) × 68200 b) Reticulum cell neoplasm type B Budding particles from the cell surface × 68200

Fig 5 a) EAC Extracellular enveloped A particles To the right one A particle (arrow) next to three C particles × 68200 b) Reticulum cell neoplasm type B Extracellular enveloped A particles × 68200

the reported finding of C-particles in our EAC tumour could explain the enhanced development of RCN following the inoculation of filtrate from EAC, other mechanisms also deserve consideration Further work is therefore necessary to establish a closer relationship of the development of RCN type B to a viral genesis

This work has received financial support from the *Norwegian Cancer Society*

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Acta path microbiol scand Sect A, 82, 578-581, 1974

A HEAT LABILE FACTOR RELATED TO THE DEVELOPMENT OF RETICULUM CELL NEOPLASMS TYPE B FOLLOWING INTRAPERITONEAL INOCULATIONS OF CELL-FREE FILTRATE OF *EHRLICH'S* ASCITES CARCINOMA

A Study of Adult, Male Mice

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A high incidence of reticulum cell neoplasms type B was recorded following repeated intra peritoneal injections of cell free filtrate prepared from *Ehrlich*'s ascites carcinoma Immunological challenge corresponding to the filtrate injections or immunological stimulation in addition to filtrate injections, did not influence the incidence or type of lymphomas to develop

Previous studies of adult female mice have demonstrated a high incidence of reticulum cell neoplasms (RCN) type B Their appearance followed subcutaneous inoculations of cell-free filtrate or vital tumour cells of *Ehrlich*'s ascites carcinoma (EAC) (*Myking* 1974) Electron microscopic examination of a subcutaneous transplant of EAC and of two RCN type B, revealed numerous virus particles in each sample Morphologically, they were identical to murine leukaemia virus types A and C (*Myking* & *Aabro* 1974) Additional factors were mentioned as possibly responsible for the enhanced development of RCN These were transfer of non-viral RNA of crude extracts from allogenic lymphocytes and the possible oncogenic effect of the antigenic material in the crude EAC filtrate used Such antigenic material will be partly derived from allogenic proteins, red cells, lymphocytes and macrophages in the ascites tumour, and partly from the EAC cells alone These have been shown to evoke immunological reactions (*Thunold* 1967 and 1968)

Development of reticulosarcomas in mice following immunological stimulation has been reported after prolonged immunological stress (*Metcalf* 1961, *Schwartz* & *Beldotti* 1965, *Cornelius* 1972, *Gleichmann* & *Gleichmann* 1973) Experiments considering the oncogenic effect of immunological stimulation of shorter duration have received little attention apart from a study by *Tyndall et al* (1972) They reported an increased incidence of reticulosarcomas in BALB/c mice following injection of sheep red blood cells twice weekly for only 12 weeks

Antigen stimulation has also been reported to modify the neoplastic process in murine

Received 11 i 74 Accepted 12 iii 74

TABLE I *Grouping, Treatment and Main Results*

	Group A	Group B	Group C
Number of animals examined	35	32	37
Age of mice at the start of the experiment (*mean ± SD in weeks*)	33 5±5 3	33 6±4 6	32 4±6 0
Treatment	Cell free EAC filtrate	Cell free EAC filtrate Heat inactivated EAC	Heat inactivated EAC filtrate
Reticulum cell neoplasms	18 (51%)	17 (53%)	9 (24%)
Age at death (mean ± SD in weeks)	86 7±24 6	86 4±20 3	93 6±24 7

leukaemia virus infections (*Babinkov & Shapiro* 1972, *Kouttab & Jutila* 1972, *Siegel & Morton* 1973)

The present study was carried out to compare the oncogenic effect of *freshly prepared EAC filtrate* with the effect of *heated filtrate* exposing the recipients to a corresponding immunological load In a separate group of mice the effect of treatment with *unheated filtrate and additional immunological stimulation* was studied

MATERIAL AND METHODS

The mice were of the same colony as that used in previous experiments (*Myking* 1974)

Cell free tumour filtrate was prepared from EAC as in previous experiments (*Myking* 1974) The tumour suspension was passed undiluted through a 0 45 μ Millipore filter after centrifugation at 1650 g for 10 minutes and after prefiltration Filtration was performed immediately before use

Inactivation of EAC and EAC filtrate was performed by heating to 60° C for half an hour The material was thereafter stored at 20° C until use

Statistics

The P values have been estimated according to the chi square test

Experimental Procedure (Table I)

Three groups A B and C, of male mice were used Three mice in group A two in group B and two in group C were later destroyed by cannibalism and therefore excluded from the experiment The final number of mice in each group and their age are given in Table I

Cell free EAC filtrate was given 4 times to the mice in groups A and B, the dose being 0 2 ml, 0 1 ml, 0 1 ml and 0 2 ml, starting at day 11 of the experiment and thereafter every other day Group C was given corresponding doses of heat inactivated filtrate

Heat inactivated EAC was given to the mice in group B 6 times, starting at day 1 of the experiment and thereafter at four day intervals, at a dose of 0 05 ml at each injection Groups A and C were given corresponding saline treatment All injections were by the intraperitoneal route The mice were kept until they died or were killed when clearly moribund Further examination, histology and classification of the lymphomas was carried out as in previous experiments (*Myking* 1974) The age at death was recorded

RESULTS

The incidence of RCN was 51 per cent and 53 per cent in groups A and B, respectively, while an incidence of 24 per cent was recorded in group C, given heat inactivated EAC filtrate The difference in incidence of RCN between groups A and C, and between groups B and C, was statistically significant (P < 0 008 for both comparisons)

The type of RCN was consistent with the type B RCN of *Dunn* (1954), and *Dunn & Deringer* (1968) No difference in the distribution of the lesions was recorded compared to previous experiments Lymphomas of other types were not recorded

The life span was a little shorter in groups A and B than in group C The difference, however, is not statistically significant No

significant difference in survival times of lymphomatous mice and non lymphomatous mice in either group was recorded

DISCUSSION

The present study of male mice confirms the previously reported findings of a high incidence of RCN type B in female mice following the inoculations of cell free filtrate of Ehrlich's ascites carcinoma The intraperitoneal route of filtrate injections as opposed to the subcutaneous used previously, did not influence the histological type of lymphomas developing, the distribution of the lesions in the organism, or the life span of the animals

A significantly lower incidence of RCN was seen following injections of heated EAC-filtrate (group C) compared to injections of unheated filtrate (group A) The RCN incidence in group C corresponds to the expected frequency of RCN developing spontaneously in this mouse colony (unpublished data) Repeated injections of heated EAC in addition to injections of unheated filtrate, did not alter the incidence of RCN, compared to the incidence following injections of unheated filtrate alone (groups A and B)

Heating to 60° C for half an hour will destroy the activity of RNA viruses (*Rich* 1968, while the antigenicity of soluble proteins is not expected to be lowered (*Cushing* & *Campbell* 1957, *Landsteiner* 1962) The activity of transplantation antigens might be expected to undergo a slight reduction (*Kandutsch* & *Reinert-Wenck* 1957), however, some transplantation antigens are known to be thermostable (*Intorp* & *Milgrom* 1968)

The high incidence of RCN type B therefore appears to depend on the presence of a heat labile factor most likely an oncogenic RNA virus Immunological challenge corresponding to the inoculations of EAC-filtrate or in addition to filtrate injections has not influenced the incidence or the type of lymphomas to develop

This work has been carried out with financial support from the *Norwegian Cancer Society* The skilled technical assistance of Mrs *Anne Marie Sundstrom* is gratefully acknowledged

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Acta path microbiol scand Sect A, 82, 582-585, 1974

HISTOLOGICAL EVALUATION OF LUNG EXPANSION IN RELATION TO VARIOUS FIXATION TECHNIQUES—IMMERSION, PERFUSION AND RAPID FREEZING

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Three alternative methods for fixation of the unopened thorax (immersion, vascular perfusion and rapid freezing) were compared in a series of newborn full term rabbits with particular reference to lung expansion and preservation of pulmonary fluid Immersion seemed to be the method of choice, since it yields acceptable fixation of both tissue and pulmonary fluid Also, the morphometric analysis indicated that postmortem collapse of the parenchyma is less prominent in the immersion fixation method than in the other two methods tested

The sponge-like structure of the pulmonary parenchyma implies some problems as regards fixation and preparation of lung specimens for histological examination, particularly when expansion patterns are to be analysed

In aerated lungs, the "elastic recoil" of the parenchyma induces some alveolar collapse as soon as the thorax is opened and the negative intrathoracic pressure is discontinued Moreover, postmortem pulmonary oedema in experimental animals has been described (M[illegible]ada 1968) Also, in any wet lung, there may be postmortem redistribution of air, under the influence of surface forces

Some of these artifacts (notably postmortem collapse redistribution of air and effusion of fluid into the alveolar compartment) would be particularly disturbing in histological studies of lung expansion and might to some extent invalidate the results of morphometric calculations based on material fixed by conventional methods

The purpose of the present study was to evaluate various fixation techniques for histological morphometric investigations of lung expansion in the newborn Particular reference was made to the influence on the pattern of alveolar expansion and the preservation of intra-alveolar fluid Only fixation techniques which could be applied to the unopened thorax were tested

MATERIAL AND METHODS

The experiments were carried out on 24 rabbit foetuses near full term The does were killed by intravenous injection of 5 ml 2M KCl on the 30th day of gestation (full term = 31 ± 1 days, mean ± SD) The abdomen was immediately opened and the uterine vessels clamped with large haemostats The rabbits to be studied before the onset of breathing (n ≈ 12) were killed in utero by intr[illegible]peritoneal injection of 0 5 ml Mebumal (sodium mebumal 60 mg/ml), the remaining animals were delivered through random uterine incisions and kept in cages at a temperature of approximately

Received 15 III 74 Accepted 15 III 74

30° C until the age of 2 hours The specimens were processed according to one of the following three alternative methods

Immersion

The unopened thorax was fixed in 10 per cent formalin for one week (*Planck* 1966)

Vascular Perfusion

After insertion of a thin cannula (Portex Flex Nylon 00) into the jugular vein the vascular bed of the animal was flushed with 1 per cent Xylocain and thereafter perfused with 200 ml 4 per cent glutaraldehyde in 0 1 M cacodylate buffer at a pressure of 100 mm Hg non pulsatile The mesenteric vessels were cut in order to drain the perfusate (*Weibel & Gil* 1968)

Rapid Freezing

The unopened thorax was rapidly freezed in liquid propane (cooled to −180° C in liquid nitrogen) and thereafter transferred to a fixative solution of absolute ethanol/acetone/glacial acetic acid 75/25/5, v/v/v picric acid, mercuric bichloride and potassium bichromate added to saturation (cf *Storey & Staub* 1962) After 6 weeks fixation at 70° C, followed by 2 weeks at −40° C, the specimen was transferred to absolute ethanol at −40° C for one week and thereafter brought to room temperature

From all lungs—regardless of the type of fixation—transverse sections were cut at the level of the cardiac ventricles and embedded in paraffin From these blocks 10 μ thick microtome sections were cut and stained with haematoxylin and eosin Besides conventional histological examination, lung volume proportions were analysed morphometrically by means of an integrating eye piece of the point-counting type (*Chalkley* 1943) The volume of the following two compartments was determined

Alveolar lumen alveoli and alveolar duct spaces

Parenchyma alveolar and bronchial walls pulmonary blood vessels and lymphatics, peribronchial and septal tissue and any other compartments of the lung which cannot be referred to as airspace

The complete transverse section of both lungs was examined in each case and the degree of alveolar expansion was calculated as follows

$$\textit{Alveolar expansion index} \quad \frac{V_{\text{alveolar lumen}}}{V_{\text{parenchyma}}}$$

RESULTS

Immersion

In animals killed in utero the alveoli were equal in size with wrinkled walls Many alveoli were filled with a finely granular material, which appeared pink in haematoxylin and eosin stainings After two hours there was patchy air expansion alternating with areas of fluid filled alveoli (Fig 1 A) From 0 to 2 hours there was a statistically significant increase of the alveolar expansion index (Table 1)

TABLE 1 *Alveolar Expansion Index of Full Term Rabbit Lungs before the Onset of Breathing and at the Age of 2 Hours after Application of Various Fixation Techniques—Immersion in Formalin, Vascular Perfusion and Rapid Freezing*

Method of fixation	No of animals	Alveolar expansion index, mean±SD 0 h	2 h
Immersion	4+4	0 39±0 11	0 84±0 29*
Perfusion	4+4	0 39±0 10	0 54±0 26
Rapid freezing	4+4	0 48±0 20	0 59±0 13

* $P < 0\ 05$

Vascular Perfusion

Uniform vascular perfusion was obviously not achieved in all specimens, since red blood cells still remained in many vessels This holds true for both 0 and 2 hour animals The lungs from the 0 hour animals had the same configuration as the 0 hour lungs fixed by immersion in formalin, however, in only few alveoli granular 'fluid' was present After two hours there was patchy alveolar expansion and minimal amounts of pulmonary fluid were preserved in unexpanded areas (Fig 1 B) The alveolar expansion index was increased from 0 to 2 hours but the difference was not statistically significant (Table 1)

Rapid Freezing

Using this method there was a good preservation of the tissue and the expansion pattern in the 0 hour animals was the same as for 0 hour animals processed according to the other methods However, only very few alveoli containing pink, finely granular pulmonary fluid remained in these lungs The expansion pattern in animals killed 2 hours

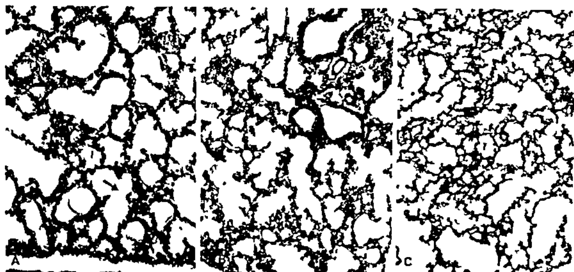


Fig 1 Lung specimens from full term newborn rabbits (age 2 hours), fixed by immersion technique (A) vascular perfusion (B) or rapid freezing (C) It applies to all methods that there is irregular alveolar expansion Air liquid interfaces are best preserved in specimens fixed by immersion (A, *arrow*) Haematoxylin and eosin × 155

after birth was similar to that after immersion or perfusion fixation (Fig 1 C) The alveolar expansion index was increased from 0 to 2 hours, although the difference was not statistically significant (Table 1)

COMMENT

Good *tissue preservation* was obtained whether immersion fixation or rapid freezing were used Vascular perfusion through extra thoracic vessels does not seem to be 100 per cent effective at least in newborn individuals, probably due to bypass of the perfusate through the ductus arteriosus These results are in accordance with observations by *Krasino et al* (1972) who used perfusion fixation via the right ventricle in their electron microscopic studies of newborn rabbits, these authors reported that this fixation was unsuccessful in animals killed before the onset of breathing

The visualization of foetal *pulmonary fluid* or the alveolar lining layer by light or electron microscopic techniques requires that the fluid carries some macromolecular complexes (e g phospholipids proteins or mucopolysaccharides) which can be precipitated by the fixative and which do not dissolve during the subsequent dehydration of the specimen In this material the preservation of pulmonary fluid is best compared in the 0 hour animals, since it is known that all alveoli are fluid filled before the onset of breathing Fluid in the airspaces of 2 hour animals might, at least theoretically, be the result of postmortem transudation It appears from the present series that the pulmonary fluid was best visualized in animals whose unopened thorax was fixed by immersion in formalin Only small amounts of fluid could be identified in lungs fixed by the other techniques

The *expansion pattern* of the lungs was similar after fixation by immersion perfusion and rapid freezing In all groups the alveolar expansion index was increased within intervals of from 0 to 2 hours however only in lungs fixed by immersion of the unopened thorax in formalin was the difference statistically significant

CONCLUSION

The immersion technique seems to be the method of choice when lung specimens are to be fixed for histological morphometric evalu

ation of lung expansion in the newborn This simple method yields an acceptable preservation of both tissue and pulmonary fluid The higher alveolar expansion index of aerated lungs fixed by immersion suggests that postmortem collapse is less prominent using this technique rather than the other two methods tested

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Acta path microbiol scand Sect A, 82, 586-588, 1974

BRIEF REPORT

IMMUNOFLUORESCENT LOCALIZATION OF ALPHA FETOPROTEIN SYNTHESIS IN ENDODERMAL SINUS TUMOR (YOLK SAC TUMOR)

G Teilum, R Albrechtsen and *B Nørgaard Pedersen*

Bergstrand & Czar (3) in 1956 described a specific alpha$_1$-fetoprotein (AFP) in serum from the human fetus, which was absent in adult sera In the human embryo a maximal concentration of AFP is found at about 12 to 13 weeks of gestation, after which it declines slowly and within a few weeks after birth is no longer detectable In 1967, *Abelev et al* (1) reported that AFP reappears not only in sera of patients with hepatomas, but also in many cases of embryonal testicular tumors It is now well-established that AFP is a marker for the cells responsible for its synthesis in early embryogenesis as well as for certain gonadal and extra gonadal germ cell tumors (6, 7), which most often have been described in indefinite terms, such as "teratoblastoma", "teratocarcinoma", "malignant teratoma" or "embryonal carcinoma", without attempts to correlate the histology of the tumors to the presence or absence of AFP It also has remained unexplained why a number of sera from patients with such germinal tumors were AFP negative

Since the site of AFP synthesis has been demonstrated to take place not only in the embryonic hepatocytes, but also in the human yolk sac at early embryogenesis (*Gitlin & Perricelli* 1970) (5) special attention has been drawn to the endodermal sinus tumor (yolk sac tumor), which was recognized and classified on a histogenetic—embryologic basis by *Teilum* in 1959 (10), and is now generally accepted as a distinct and specific entity According to *Teilum's* histogenetic classification (11, 12) these germ cell tumors represent a selective differentiation of a multipotent 'embryonal carcinoma' stem cell into tumors of the extra embryonic fetal yolk sac endoderm *Teilum* also pointed out that apart from pure forms of endodermal sinus tumors (most often found in young women and children, and more rarely in the adult testis) some of the variegated histological patterns of 'embryonal carcinoma" and "teratocarcinoma' actually represent proliferation of fetal yolk sac endoderm (11, 12)

There are already a few reports of AFP in serum in patients with tumors diagnosed as specific endodermal sinus tumor (2, 13, 14)

The histogenetic interpretation of these germ cell tumours in accordance with the available clinical and experimental observations would explain the reappearance of AFP as being dependent on the presence of a significant vitelline component producing AFP, analogous with AFP synthesis of the fetal yolk sac in early embryogenesis Immunofluorescent study of the localization of AFP in transplantable murine teratocarcinomas (*Engelhardt et al* (4) most frequently showed a bright AFP specific fluorescence in epithelium of endodermal origin lining cysts and tubules

The purpose of the present study is to demonstrate the AFP producing cells in tissue sections from a pure endodermal sinus tumor by means of indirect immunofluorescence technique

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Received 7 v 74 Accepted 7 x 74

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Sections from the endodermal sinus tumor

Fig 1 Endodermal sinus structures with mantling of vessels by yolk sac endoderm (H & E × 130)

Fig 2 Endodermal sinus showing prominent intra and extracellular PAS positive, *hyaline globules* (PAS × 130)

Fig 3 Indirecte immunofluorescence AFP staining Same area as fig 1 An endodermal sinus structure with a bright green granular staining of the lining cells (× 430)

Fig 4 Indirecte immunofluorescence AFP staining A bright green staining is found in the intra and extracellular hyaline globules (× 430)






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Acta path microbiol scand Sect A, 82, 586 588, 1974

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Acta path microbiol scand Sect A, 82 589 604, 1974

STUDIES OF THE ACTIVITY IN CHICKEN SERUM THAT STIMULATES THE LYMPHOID DEVELOPMENT OF THE EMBRYONIC CHICKEN THYMUS IN ORGAN CULTURE

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Some properties of the thymus stimulating activity in chicken serum (TSAS) which is essential for the lymphoid development of the embryonic chicken thymus in organ culture, have been studied and a partial purification has been performed TSAS resisted heating for 30 min at 60° C but not higher temperatures During dialysis and ultrafiltration of serum a loss of TSAS down to a constant level of about 50 per cent of the original activity was obtained TSAS was sensitive to treatment at pH 5 or lower but resisted alkaline pH up to at least pH 10 When serum was fractionated with ammonium sulphate little TSAS precipitated at 2 43 M ammonium sulphate or lower At higher concentrations TSAS was found in the precipitates, but even at 4 05 M significant activity remained in the supernatant TSAS was recovered from a fraction dominated by the serum albumins after gel filtration on Sephadex G 200 and G 100 Further fractionation by ion exchange chromatography on DEAE Sephadex separated the TSAS from serum albumin These results suggest that part of the TSAS is associated with one or more specific factors with a molecular weight of approximately 70 000 The effect of the partly purified TSAS on the growth and morphologic development of the embryonic thymus in organ culture was studied

One important function of the thymus as a central lymphoid organ is the production of lymphoid cells with specific functional characteristics By seeding these cells to the peripheral lymphoid organs (8, 10, 18) and apparently also by secreting a humoral lymphopoietic factor (9, 12, 28) the thymus influences the development and maintenance of the morphology and function of peripheral lymphoid organs

The external and internal regulation of thymic development and lymphopoiesis is poorly understood However, there is evidence that an external control of the thymus is accomplished by mediators from the pituitary gland, either directly or indirectly via other endocrine organs such as the thyroid gland (22) Pituitary growth hormone exerts a thymotropic effect either alone or together with thyroxine and also influences the peripheral lymphoid organs (13, 19, 22) A specific thymotropic hormone of pituitary origin has been suggested but not yet identified (5) Whether the target cells of this pituitary control are the epithelial or lymphoid components of the thymus or both is not

Received 15 ii 74 Accepted 15 iii 74

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known A feed back mechanism between the thymus and the pituitary gland has also been indicated (2, 21, 22) The inhibitory action of glucocorticosteroid hormones on thymic lymphopoiesis is well documented (3) Regulatory effects of sex hormones on the thymus have also been suggested (6, 7) Recently thyrocalcitonin has been shown to inhibit and parathormone to stimulate thymic lymphopoiesis (14)

The evidence for an autonomous internal regulation of the lymphopoiesis in the thymus, in particular the importance of the thymus epithelium in this, have been reviewed by *Metcalf* (15, 16) and *Metcalf & Moore* (17)

We have previously described an organ culture system for the embryonic chicken thymus (1) This organ culture system appears suitable for the examination of potential thymotropic factors It permits the reproducible morphological development of thymic anlagen from a stage with only relatively few lymphoid precursor cells to a stage containing large numbers of predominantly small lymphoid cells (1, 24, 26) We have also presented results indicating that the lymphoid cells differentiating in the organ cultures, are able to react to lymphocyte mitogens *in vitro* (24, 27)

The organ culture technique included a complex culture medium, RPMI 1640, supplemented with heat inactivated chicken serum only Data showing that the chicken serum was essential for both initiation and maintenance of lymphoid development in the thymic anlagen have been presented (26) This thymus growth stimulating activity in serum (TSAS) may be due to specific factors of physiological significance The purpose of the present investigation was therefore to define some of the properties of the stimulating activity in serum necessary for the lymphoid development of the embryonic thymus in organ culture Parts of these results have been briefly reported elsewhere (27)

MATERIALS AND METHODS

Organ culture technique The White Leghorn chicken embryos were of DeKalb Chix strain 161 or Babcock B 300 Details of the incubation conditions for the eggs, the procurement of the thymic anlagen from 10 day-old embryos and the organ culture technique have been given elsewhere (1, 26), The culture medium in the present investigation was RPMI 1640 (Flow Laboratories, Irvine U K) with supplements as indicated Heat inactivated chicken serum (CS), 10 per cent (v/v) in the medium, was used as a positive control in all experiments The culture time was six days where not otherwise indicated Medium changes were not performed because they did not improve the lymphoid development of the anlagen

Evaluation of lymphoid development The effect of the different medium supplements on the lymphoid development of the thymic anlagen was determined, as previously described (26), by counting the number of lymphoid cells obtained from homogenized thymic anlagen Four cultures were used for each treatment These were either pooled at harvest or harvested separately and the cell yield was expressed as the mean number of cells per anlage

Chicken serum The procurement of serum from 5 8 weeks old chickens (CS) has been dealt with elsewhere (26) Whole serum and the various serum preparations were stored at −85° C

Heat inactivation The serum was usually heat inactivated at 56° C for 30 minutes To examine the heat resistance of the thymus stimulating activity, CS was incubated at respectively 56, 60, 80 and 100° C for 30 minutes After centrifugation any precipitate was discarded and the supernatant collected

Sterile filtration Whole serum was always aseptically handled and needed no further sterilization If necessary, sterilization of serum preparations was done by filtration through 0 45 μ Millipore filters

Dialysis Whole serum or fractions of serum were dialysed in Visking 23/32 cellulose dialysis tubing (Union Carbide, New York, N Y U S A) for 2 days against four changes of 100 fold volumes of phosphate buffered saline (PBS), pH 7 4 (0 05 M sodium phosphate and 0 09 M NaCl) The dialysis tubing is assumed to retain molecules with a Mw larger than 6,000 (4)

Test of different concentration procedures Concentration of diluted serum was in four experiments performed by ultrafiltration through an SM 16 305 collodion membrane (Sartorius Membranfilter, Gottingen, Germany) This membrane retains molecules with a Mw larger than 12 000 In five experiments the concentration procedure was carried out by ultrafiltration through Diaflo UM 2 or UM 10 filters (Amicon Lexington Mass U S A) with cut off levels of 1,000 and 10 000 respectively In two other experiments the concentration was performed by lyophilization

Concentration of serum fractions obtained at the different separation steps was performed by ultra

filtration through either collodion membranes or UM 10 filters.

Test of pH sensitivity. The pH sensitivity of TSAS was tested by incubating samples of heat inactivated CS for 30 min with an equal amount of buffer of pH values ranging from 2 to 10. The following buffers in 0.1 M concentration were used to achieve the desired pH: Citrate-HCl, pH 2–4, citrate phosphate, pH 5 and 6, glycine-NaOH, pH 9 and 10. The pH of the buffer serum mixture was always checked. After the incubation the sera were centrifuged, the supernatants dialysed against PBS, reconcentrated to the original serum volume, sterile filtered and tested as medium supplements in organ culture in two experiments.

Ammonium sulphate precipitation. Fractionation of chicken serum by sequential precipitation of protein from serum half diluted with PBS, pH 7.0, was performed with ammonium sulphate ($(NH_4)_2SO_4$) at a final concentration of 1.50, 1.66, 2.03, 2.43, 3.08 and 4.05 M at 4° C under continuous stirring for two hours. At each step both precipitates and supernatants were collected after centrifugation. The supernatants were concentrated to half the original serum volume, dialysed and restored to the original serum volume with PBS. The precipitates were washed once with the $(NH_4)_2SO_4$ solution and were then redissolved in PBS, dialysed and returned to the original serum volume.

Gel filtration on Sephadex G 200. Whole serum was fractionated by gel filtration at 12° C on Sephadex G-200 using a K 25/100 column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with PBS, pH 7.4. Five ml fractions were collected after elution by upward flow at a rate of 3.5 ml/cm² × h. After determination of the protein concentration, fractions were pooled as indicated in Fig. 4 and concentrated to the original serum volume applied to the column.

Gel filtration on Sephadex G 100. The supernatant from chicken serum precipitated with 2.43 M $(NH_4)_2SO_4$ as described above, was concentrated twice. It was then simultaneously desalted and separated by gel filtration on Sephadex G 100 using a K 50/100 column (Pharmacia). The gel bed was equilibrated with PBS, pH 7.4. Ten ml fractions were collected by downward elution at a flow rate of 5.1 ml/cm² × h. Fractions were pooled as indicated in Fig. 5 and concentrated to about 1.6 times the original serum volume.

Chicken serum supernatants after $(NH_4)_2SO_4$ precipitation at 2.43 M were also separated on a large scale on Sephadex G 100, using a K 100/100 column (Pharmacia) equilibrated with 0.05 M tris HCl buffer, pH 8.0, ionic strength 0.10. One fraction, corresponding to the second peak (the 'albumin peak') in the elution diagram (Fig. 5), was saved. It was concentrated to approximately the original serum volume for further separation.

Ion exchange chromatography. The 'albumin peak' material from the gel filtration on Sephadex G 100 was separated by ion exchange chromatography on DEAE Sephadex A 50 (Pharmacia). The ion exchange resin was equilibrated in 0.05 M tris HCl buffer, with 0.073 M NaCl, pH 8.0, ionic strength 0.10 (2.73 mho). A sample containing approximately 1 g protein in the same buffer was applied to 100 ml resin in a K 25/50 column (Pharmacia). The column was eluted with 500 ml starting buffer followed by two continuous linear gradients of 0.05 M tris HCl buffer with increasing salt concentration. The first gradient was formed from the starting buffer and buffer with 0.17 M NaCl, ionic strength 0.20 and the second from the latter buffer and buffer with 0.475 M NaCl, ionic strength 0.50. Fractions of 3 ml were collected at a flow rate of 40 ml/h. After determination of the protein concentration and the conductivity, the fractions were pooled as indicated in Fig. 6, concentrated to 20 mg protein per ml, dialysed against PBS, and tested in organ culture as medium supplements.

Protein determination. The protein concentration in serum and serum fractions was determined from the absorbance at 280 nm using bovine serum albumin in distilled water as standard.

Immunoelectrophoresis. The various serum fractions were analysed by immunoelectrophoresis in agar, using an LKB 6800 A immunoelectrophoresis equipment (LKB, Stockholm, Sweden). Rabbit antiserum to whole chicken serum served as developing reagent.

Expression of the thymus stimulating activity. The thymus stimulating activity (TSAS) in different serum fractions and in serum after various treatments was related to that of heat inactivated (56° C, 30 min) whole chicken serum in every experiment and expressed as the mean number of cells per anlage obtained with the test material in per cent of the number of cells obtained per anlage with the 10 per cent control CS.

Student's t test was used to evaluate the significance of the difference between group means. The calculations were performed on the number of lymphoid cells per thymic anlage after logarithmic transformation.

Morphological examination. The morphological development of thymic anlagen cultured with different medium supplements was examined in smears and sections.

Smears made from thymic cells suspended in 50 per cent CS were air dried, fixed in methanol

fixed in an ice-cold mixture of 1 per cent formaldehyde and 2 per cent glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, and embedded in Epon. Sections for light microscopy, 1 μ thick, were stained with 1 per cent toluidine blue in an

TABLE 1 *The Effect of Heating for 30 min on the Thymus Stimulating Activity of Chicken Serum*

Temperature (° C)	Protein conc (mg/ml)	Stimulatory activity (%)*
–	51	76 0±4 7 (6)
56	51	100 0 (6)
60	51	74 7 (2)
80	20	4 3 (2)
100	13	7 4 (2)

* Expressed as per cent of the thymus stimulating activity of chicken serum heat inactivated at 56° C for 30 min (mean ± S E M) Figures within parenthesis represent the number of experiments

aqueous solution of 1 per cent borax Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined in a Jeol JEM 100 B *electron microscope*

RESULTS

Heat inactivation · The effect of heating at different temperatures on the thymus stimulating activity in serum (TSAS) was tested in two complete experiments Four further experiments were performed to specifically study the effect of heat inactivation at 56° C for 30 min As illustrated in Table 1 heat inactivation of CS at 56° C for 30 min caused a significant increase of the TSAS (t = 4 75, d f = 5, P < 0 01) Heating at 60° C resulted in a loss of activity in comparison with heating at 56° C but not when related to untreated serum After heating at 80° C and 100° C, protein precipitates were formed which decreased the protein concentration to approximately 40 per cent and 25 per cent, respectively, of the original concentration The TSAS decreased proportionally more than the total protein concentration Thus TSAS was relatively thermo stable up to 60° C but was inactivated or precipitated at still higher temperatures

pH-sensitivity Treatment of CS between pH 2 and 10 gave only insignificant protein precipitation The effect on TSAS was tested in one experiment

As demonstrated in Fig 1 serum treated at pH 6, 9 or 10 showed the same TSAS The number of cells obtained was 63 per cent of the number of cells obtained by the usual 10 per cent CS control Treatment of CS at pH 5 resulted in a significant loss of TSAS as compared to pH 6 (t = 2 55 d f = 6, P < 0 05) Further decrease of the pH resulted in a significant successive decrease of the TSAS The number of cells obtained by CS treated at pH 2 was only 18 per cent of the number obtained by CS treated at pH 6

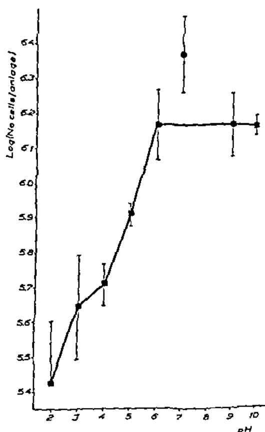


Fig 1 Lymphoid cell yield (log [no cells/anlage]) of thymic anlagen cultured in medium supplemented with untreated CS (●) or CS treated at different pH between 2–10 (■) Each point represents the mean ± S E M of four cultures

Effects of dialysis and concentration These experiments are summarized in Table 2 Dialysis against PBS and ultrafiltration (Collodion and Diaflo membranes) resulted in a marked decrease in TSAS varying between experiments but usually in the order of 45

TABLE 2 *The Effect of Dialysis and Different Concentration Procedures on the Thymus Stimulating Activity of Chicken Serum (CS)*

Treatment	Number of experiments	Stimulatory activity (%)*
Sterile filtration		
Millipore, 1 2 and 0 45 μ membranes	1	117 1
Ultrafiltration§		
Amicon UM 2 membrane	1	64 3
Amicon UM 10 membrane	3	57 2 ± 3 1
Collodion membrane	4	48 1 ± 11 7
Repeated ultrafiltration§	1	
Amicon UM 10 membrane 1st filtration		61 4
2nd		41 0
3rd "		40 7
Lyophilization		
CS, 10 × dilution with distilled water	3	92 3 ± 26 2
CS, undiluted	2	90 3
Dialysis		
2 days against PBS	5	60 1 ± 7 0
Repeated dialysis	1	
17 hours against PBS 1st dialysis		83 7
2nd ,		65 2
3rd ,		65 2

* Per cent of the thymus stimulating activity of untreated CS control The figures represent mean ± S E M

§ Reconcentration after 10 × dilution of CS with PBS

per cent Filtration through a Millipore membrane or lyophilization of diluted or undiluted CS gave no significant loss of TSAS

Effect of dialysis time To test for the time dependent loss of active material during dialysis the following type of experiment was performed

Eight 5 ml samples of heat inactivated CS were dialysed under sterile conditions at 4° C against a 400 fold volume of PBS The dialysis time was 0, 9 18 29 hours and 2 3 4, and 5 days All samples were kept at 4° C until the last dialysis was terminated The volumes of the samples before and after dialysis did not differ They were therefore tested in organ culture directly

The results of three experiments are demonstrated in Fig 2 There was a significant loss of about 50 per cent of the original activity during the first 2 days of dialysis The activity remained approximately constant at this level or even increased slightly until day 5 No difference in activity of control CS stored at 4° C and at −85° C during the dialysis period could be detected

Repeated dialysis and ultrafiltration Repeated dialysis and repeated ultrafiltration was performed to test whether further losses of the TSAS, remaining after one dialysis or ultrafiltration, occurred

A 15 ml sample of heat inactivated CS was dialysed for 17 hours against a tenfold volume of PBS at 4° C Ten ml of the dialysed CS were transferred to a new dialysis bag and were dialysed for another 17 hour period The remaining 5 ml were stored at 4° C The procedure was repeated once more The samples of dialysed CS were tested as medium supplements in one experiment

Table 2 shows that there was a 35 per cent decrease of TSAS during the first two 17-hour dialysis periods The third dialysis did not result in a further loss of activity

Fifteen ml of heat inactivated CS were diluted 10 × with PBS and reconcentrated to the original volume by ultrafiltration through a UM 10 membrane Five ml were collected and the remaining 10 ml were diluted and reconcentrated as above The procedure was repeated once more and the three samples of ultrafiltrated CS were tested as medium supplements in one experiment

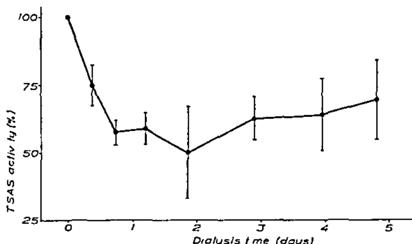


Fig 2 Time dependent loss of thymus stimulating activity in serum during dialysis The activity of dia lysed serum was related to the activity of untreated serum (%) Each point represents the mean ± S E M of three experiments

Table 2 shows that one ultrafiltration resulted in a loss of TSAS of 39 per cent After a second filtration the activity decreased by another 20 per cent The third ultrafiltration did not result in any further decrease of TSAS

Recombination of ultrafiltrate and concentrate To test for thymus stimulating material in the ultrafiltrate of CS the following experiment was performed

100 ml CS was concentrated 6 5 times by ultra filtration through a UM 10 membrane Part of the concentrate was reconstituted to its original volume with either filtrate or RPMI 1640 Such recon stituted concentrate and the filtrate were tested as medium supplements in organ culture in two ex periments The effect of filtrate in combination with 0 625 per cent and 10 per cent CS as medium supplements was also tested in one experiment.

The results are shown in Table 3 The observed 33 per cent decrease of the TSAS in the concentrated CS was not statistically significant as compared to untreated CS ($t = 1\ 81$, $d\ f = 14$, $P > 0\ 05$) The filtrate showed no stimulating activity Recombination of ultrafiltrate and concentrate gave a small, but not significant, increase of the thymus stimulating activity Furthermore there was no significant potentiating effect of filtrate on whole 0 625 per cent or 10 per cent CS

Ammonium sulphate precipitation Table 4 gives the results from one of 3 $(NH_4)_2SO_4$

TABLE 3 *Test for the Presence of TSAS in Ultrafiltrate of CS*

Medium supplement	^{10}log (no cells/anlage)*	Stimulatory activity (%)§
10 % concentrate	5 99±0 06 (8)	67 1
10 % filtrate	3 59±0 08 (8)	0 3
10 % concentrate + filtrate	6 06±0 05 (8)	77 8
0 625 % CS	4 89±0 31 (4)	4 7
0 625 % CS + 10 % filtrate	4 52±0 44 (4)	2 0
10 % CS + 10 % filtrate	6 36±0 08 (4)	140 3

* Mean ± S E M no of cultures given within parenthesis
§ Per cent of the thymus stimulating activity of untreated CS control

TABLE 4 *Recovery of the Thymus Stimulating Activity after Ammonium Sulphate Fractionation of Chicken Serum (CS)*

Fraction	Protein conc (mg/ml)*	Stimulatory activity (%)§
Diluted and conc CS	32	56 5
1 50 M precipitate	12	3 4
1 50 M, supernatant	20	46 6
2 43 M, precipitate	9	9 9
2 43 M, supernatant	18	75 7
4 05 M, precipitate	10	52 9
4 05 M, supernatant	3	25 9

* Protein concentration of untreated CS was 40 mg/ml

§ Per cent of the thymus stimulating activity of untreated CS control

fractionation experiments The volumes of the different fractions were equal to that of the original serum sample The protein con centrations of each fraction are also shown in the table The two controls included were 10 per cent heat inactivated CS and 10 per cent of CS dialysed diluted and concentrated in the same way as the fractions from the precipitation procedure The latter control showed about 50 per cent of the thymus stimulating activity of the former The pre cipitates formed at a final concentration of 1 50 M $(NH_4)_2SO_4$ did not contain any TSAS activity The latter remained in the supernatant The same result was obtained in other experiments, not shown in the table, using 1 66 M and 2 03 M $(NH_4)_2SO_4$ Using 2 43 M $(NH_4)_2SO_4$, a small part of the ac tivity was found in the precipitate At the same time the activity appeared to increase in the supernatant. Using 4 05 M $(NH_4)_2SO_4$ (100 per cent saturation), the main part of the TSAS was found in the precipitate, but significant activity still remained in the super natant

The dose response curve for CS depleted of γ-globulins by $(NH_4)_2SO_4$ precipitation at 1 42 M is demonstrated in Fig 3 which shows the number of lymphoid cells per thymic anlage as a function of the ²log (concentration of agamma CS) in two experiments The curve has approximately the same shape as that for whole chicken serum described previously (26) It appears to be biphasic and is approximately linear at higher serum concentrations

Gel filtration on Sephadex G 200 Chicken serum was fractioned on Sephadex G 200 The elution pattern of protein is shown in Fig 4 Four peaks were obtained The eluted material representing the four peaks were pooled as indicated in the figure The four fractions were concentrated and tested as culture medium supplements in three separate experiments Controls in these experiments were 10 per cent heat inactivated CS and CS diluted with elution buffer and reconcentrated by collodion membrane ultrafiltration The lower part of Fig 4 demonstrates the thymus stimulating activity in the four fractions The main part 42 ± 6 per cent of the activity of the 10 per cent CS control, was found in fraction three Fraction two contained 9 ± 4 per cent, while fractions one

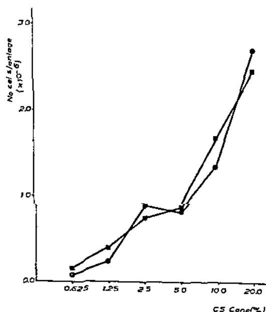


Fig 3 Effect of the concentration of chicken serum precipitated with 1 42 M $(NH_4)_2SO_4$ on the lymphoid cell yield (No cells/anlage) of thymic anlagen in organ culture (two experiments) Each point represents the mean of four cultures.

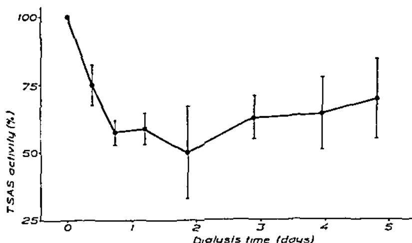


Fig 2 Time dependent loss of thymus stimulating activity in serum during dialysis The activity of dialysed serum was related to the activity of untreated serum (%) Each point represents the mean ± S E M of three experiments

Table 2 shows that one ultrafiltration resulted in a loss of TSAS of 39 per cent After a second filtration the activity decreased by another 20 per cent The third ultrafiltration did not result in any further decrease of TSAS

Recombination of ultrafiltrate and concentrate To test for thymus stimulating material in the ultrafiltrate of CS the following experiment was performed

100 ml CS was concentrated 6 5 times by ultra filtration through a UM 10 membrane Part of the concentrate was reconstituted to its original volume with either filtrate or RPMI 1640 Such reconstituted concentrate and the filtrate were tested as medium supplements in organ culture in two experiments The effect of filtrate in combination with 0 625 per cent and 10 per cent CS as medium supplements was also tested in one experiment.

The results are shown in Table 3 The observed 33 per cent decrease of the TSAS in the concentrated CS was not statistically significant as compared to untreated CS ($t = 1\ 81$; d f $= 14$, $P > 0\ 05$) The filtrate showed no stimulating activity Recombination of ultrafiltrate and concentrate gave a small, but not significant, increase of the thymus stimulating activity Furthermore there was no significant potentiating effect of filtrate on whole 0 625 per cent or 10 per cent CS

Ammonium sulphate precipitation Table 4 gives the results from one of 3 $(NH_4)_2SO_4$

TABLE 3 *Test for the Presence of TSAS in Ultrafiltrate of CS*

Medium supplement	^{10}log (no cells/anlage)*	Stimulatory activity (%)§
10 % concentrate	5 99±0 06 (8)	67 1
10 % filtrate	3 59±0 08 (8)	0 3
10 % concentrate + filtrate	6 06±0 05 (8)	77 8
0 625 % CS	4 89±0 31 (4)	4 7
0 625 % CS + 10 % filtrate	4 52±0 44 (4)	2 0
10 % CS + 10 % filtrate	6 36±0 08 (4)	140 3

* Mean ± S E M no of cultures given within parenthesis

§ Per cent of the thymus stimulating activity of untreated CS control

TABLE 4 *Recovery of the Thymus Stimulating Activity after Ammonium Sulphate Fractionation of Chicken Serum (CS)*

Fraction	Protein conc (mg/ml)*	Stimulatory activity (%)§
Diluted and conc CS	32	56 5
1 50 M, precipitate	12	3 4
1.50 M, supernatant	20	46 6
2 43 M, precipitate	9	9 9
2 43 M, supernatant	18	75 7
4 05 M, precipitate	10	5? 9
4 05 M, supernatant	3	25 9

* Protein concentration of untreated CS was 40 mg/ml

§ Per cent of the thymus stimulating activity of untreated CS control

fractionation experiments The volumes of the different fractions were equal to that of the original serum sample The protein con centrations of each fraction are also shown in the table The two controls included were 10 per cent heat inactivated CS and 10 per cent of CS dialysed, diluted and concentrated in the same way as the fractions from the precipitation procedure The latter control showed about 50 per cent of the thymus stimulating activity of the former The pre cipitates formed at a final concentration of 1 50 M $(NH_4)_2SO_4$ did not contain any TSAS activity The latter remained in the supernatant The same result was obtained in other experiments, not shown in the table, using 1 66 M and 2 03 M $(NH_4)_2SO_4$ Using 2 43 M $(NH_4)_2SO_4$ a small part of the ac tivity was found in the precipitate At the same time the activity appeared to increase in the supernatant Using 4 05 M $(NH_4)_2SO_4$ (100 per cent saturation), the main part of the TSAS was found in the precipitate, but significant activity still remained in the super natant

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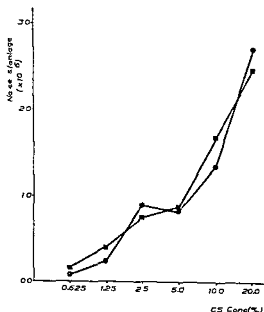
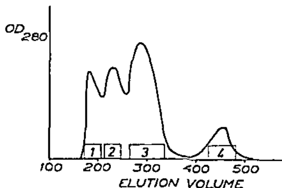


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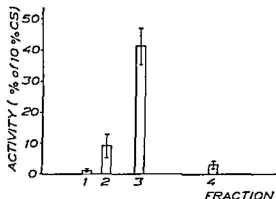


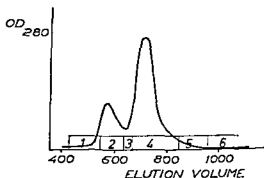
Fig 4 Elution pattern of chicken serum after gel filtration on Sephadex G 200 The four fractions (I–IV), tested as medium supplements in organ cultures are indicated

The lower part of the figure shows the thymus stimulating activity of the fractions related to the activity of whole CS (%) Mean ± S E M of three experiments Whole serum diluted and reconcentrated in the same way as the serum fractions showed 33 per cent activity

and four showed little activity, 1 ± 0 5 per cent and 4 ± 1 per cent respectively The diluted and reconcentrated control serum demonstrated a loss of 67 per cent of the TSAS

Gel filtration on Sephadex G-100 The supernatant from chicken serum precipitated with 2 43 M $(NH_4)_2SO_4$ resolved into two large protein peaks after gel filtration on Sephadex G 100 (Fig 5) The main constituent of the first peak was probably haptoglobin and the second peak albumin, as revealed by immunoelectrophoresis The eluant was pooled into six fractions as shown in Fig 5 and each was tested as medium supplement in organ culture Thymus stimulating material was recovered from fraction four which corresponded to the albumin containing peak in the elution pattern All other fractions, including the haptoglobin fraction, showed little stimulating activity The active fraction four contained transferrin and at least four other minor components besides albumin, as revealed by immunoelectrophoresis

Ion exchange chromatography The albumin containing fraction from the separation on Sephadex G-100 (fraction 4) was further separated by ion exchange chromatography on DEAE-Sephadex A50 in four experiments The material resolved into three peaks (Fig 6 A) The results of the immunoelectrophoretic analysis (Fig 7) revealed that



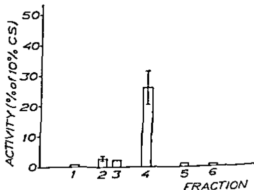


Fig 5 Elution pattern after gel filtration on Sephadex G 100 of the supernatant from whole chicken serum precipitated with 2 43 M $(NH_4)_2SO_4$ The six indicated fractions (I VI) were tested as medium supplements in organ culture

The lower part of the figure shows the thymus stimulating activity related to the activity of whole CS (%) One experiment for fractions I, III, V and VI mean ± S E M of three experiments for fractions II and IV

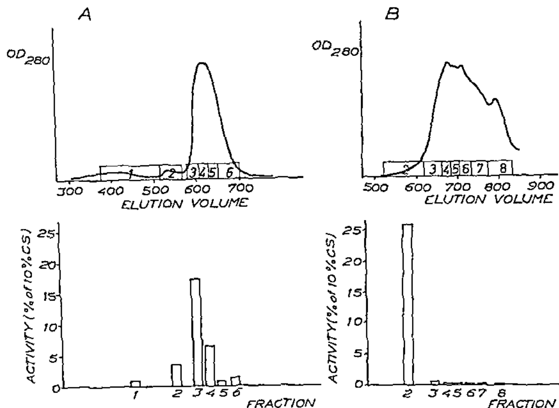


Fig 6 A Elution pattern after separation on DEAE Sephadex A50 of CS fraction IV from Sephadex G 100 fractionation The fractions tested in thymic organ culture are indicated in the figure The thymus stimulating activity of the different fractions in per cent of the activity of whole CS is given in the lower part of the figure

B Same as Fig 6 A but with a broadened elution gradient. The first fraction, containing transferrin, excluded from the figure

the first was dominated by transferrin and the third by albumin The second contained 4–5 unidentified components but was virtually free of albumin The material from these separations was tested in organ culture in 6 experiments

Fig 6 A shows the result of one typical experiment. The transferrin peak (fraction 1) consistently failed to stimulate thymic growth The second peak (fraction 2) showed a small but significant stimulating activity, but this was preferentially located to the first two fractions (3 and 4) of the albumin peak The remaining fractions (5 and 6) of this peak showed significantly less activity Fig 6 B shows that broadening of the eluting gradient over peak 3 resulted in a separation of the thymus stimulating activity from the albumin

The effect of varying the concentration of the active fraction (fraction 3 in Fig 6 A or fraction 2 in Fig 6 B) on the development of the thymus anlagen in organ cultures was studied The fraction contained 20 mg protein per ml and was tested in concentrations ranging from 1 25 to 20 per cent (v/v) in the culture medium Three experiments were done

The results of one of the two experiments using fraction 2 in Fig 6 B is shown in Fig 8 The number of lymphoid cells per anlage increased in an almost linear fashion with the logarithmic increase of the concentrations of

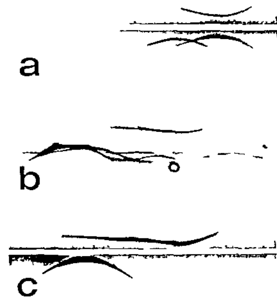


Fig 7 Immunoelectrophoretic patterns of CS fractions from separation on DEAE Sephadex A50 (a) Upper well first and lower well second half of the transferrin peak (fraction 1 Fig 6 A) (b) Upper well active, albumin free fraction (fraction 2 Fig 6 B) compared to whole CS in lower well (c) Upper well active albumin free fraction (fraction 2 Fig 6 B) lower well inactive albumin fraction (fraction 3 in Fig 6 B)

the serum fraction The mean cell yield at 20 per cent concentration was 44 per cent of that obtained by 10 per cent CS as medium supplement

The time dependent development of the thymus in organ cultures supplemented with 10 per cent of the active albumin free fraction (fraction 2 of Fig 6 B) was studied in vivo experiments The fraction contained 20 mg protein per ml As shown in Fig 9, CS and the fraction gave both a rapid increase in the number of lymphoid cells per anlage between day 0–6 With the fraction the number of cells reached a maximum on day 6 The decrease in cell number during the following 6 days (6–12) was not significant ($t = 1\ 45$), however With CS the increase of cells continued and a peak value was obtained on day 9 The number of cells per anlage did not differ significantly between anlagen cultured with CS or the fraction for 3 days but was significantly higher with CS on day 6 ($t = 2\ 47$, $d\,f = 6$, $P < 0\ 05$) and onwards

Morphological examination Virtually all cells obtained from the thymus anlagen through homogenization were typical lymphoid cells when examined in MGG stained smears

The morphology of thymus anlagen developed *in vivo* or in organ cultures for 8 days was compared The culture medium was supplemented either with whole CS the active albumin free fraction (fraction 2 Fig 6 B) or with an inactive albumin fraction (fraction 5 Fig 6 B) from the ion exchange chromatography separation The active fraction or whole CS supported the *in vitro* development of the initially mainly epithelial anlage into a typical lymphoid tissue with large numbers of lymphocytes (Fig 10 a c) The morphology corresponded to that *in vivo* (Fig 10 d) although the anlagen were considerably smaller and less densely populated by lymphoid cells No lymphoid development occurred with the inactive fraction instead an atrophy of the epithelial anlagen was observed (*Fig 10 b*)

Electron microscopical studies (Fig 11 a–d) demonstrated that in the anlagen cultured with the active fraction (Fig 11 a) or whole CS (Fig 11 c) both the epithelial and lymphoid cells were morphologically well preserved However the mitochondria were often swollen and vacuolized and central areas with degenerative changes were noted in the anlagen The typical lymphoid cells in these cultures appeared on the average larger containing more cytoplasm than the lymphocytes developed *in vivo* (Fig 11 d) Anlagen cultured with the inactive fraction completely lacked lymphoid cells and vacuolized and pycnotic cells were frequently found (Fig 11 b)

DISCUSSION

Previous work demonstrated that the presence of chicken serum was necessary for the

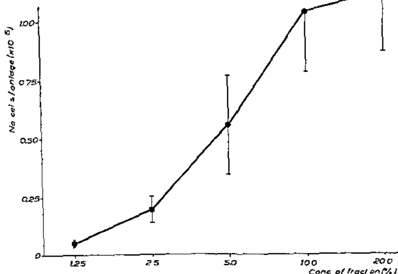


Fig 8 Effect of the concentration of the active albumin free fraction from DEAE Sephadex A50 separation (fraction 2, Fig 6 B) on the lymphoid cell yield (No cells/anlage × 10 ⁶) of thymic anlagen in organ culture Each point represents the mean ± S E M of four cultures

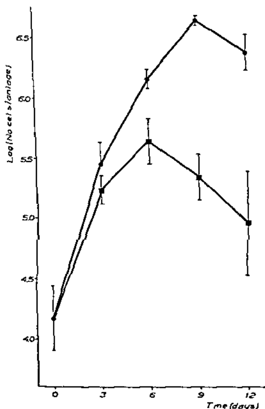


Fig 9 Time dependent lymphoid development (Log [No cells/anlage]) of thymic anlagen in organ cultures supplemented with 10 per cent active albumin free fraction from DEAE Sephadex A50 separation (fraction 2, Fig 6 B) (■) or 10 per cent whole CS (●) Each point represents the mean ± S E M of four cultures

lymphoid development of the embryonic chicken thymus in organ culture (1, 26) The present study explored the possibility that the thymus stimulating activity in serum (TSAS) might be due to one or several definable serum factors

The results show that approximately 50 per cent of the total TSAS was lost during dialysis and ultrafiltration The loss was not further increased by prolonged dialysis, or by repeated dialysis or ultrafiltration This may indicate that the TSAS is due to material of both higher and lower Mw than 6,000–12,000, which represents the retention limits for the dialysis and ultrafiltration membranes used It is perhaps more likely that the loss of TSAS represents inactivation of material of high Mw, rather than escape of material of

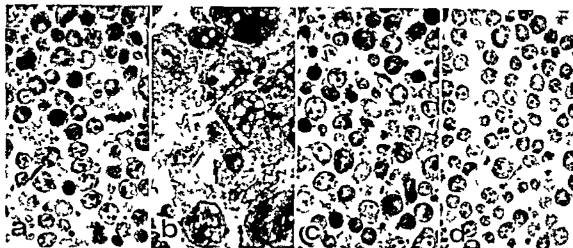


Fig 10 Thymic anlage of 10 day old chicken embryo after 8 days in organ culture supplemented with (a) Active albumin free CS fraction (fraction 2, Fig 6 B) from separation on DEAE Sephadex A50 (b) Inactive albumin containing fraction (fraction 5, Fig 6 B) from same separation, (c) Whole CS By way of comparison the thymus of the 18 day old chicken embryo is shown (d) Toluidine blue (900 ×)

low Mw, because no active material was recovered in the ultrafiltrate outside the membrane *Picz et al* (23) demonstrated a considerable autoproteolytic activity in dialysed human serum probably due to enzyme inhibitors of low Mw escaping from the serum during the dialysis A similar mechanism may account for the great loss of TSAS during ultrafiltration or dialysis in the present investigation

The TSAS was relatively resistant to heating at 60° C for 30 minutes, but was destroyed at 80° C and 100° C In fact heating at 56° C significantly increased the activity This indicated that the essential thymus stimulating activity is neither associated with a markedly heat stable nor heat labile material

Treatment of serum at pH 2–10 resulted in decreased TSAS below pH 6 This suggests that TSAS is sensitive to acid but resistant to alkaline pH values

Because a dialysis step was used to neutralize the acidified sera however it could not be determined whether the TSAS actually was destroyed or an active low Mw component was dissociated and lost through the dialysis membrane

The high Mw material showing TSAS precipitated at a concentration of $(NH_4)_2SO_4$ higher than 2 43 M (60 per cent saturation), although some activity remained in the supernatant even at 4 05 M (100 per cent saturation) Therefore the β and γ globulins appear to lack thymus stimulating activity This is also suggested by the results of the fractionation of whole serum on Sephadex G 200, where all TSAS lodged in the albumin fraction The TSAS was also found in the albumin peak, with little activity in the haptoglobulin fraction, after Sephadex G 100 fractionation of the serum supernatant from 2 43 M $(NH_4)_2SO_4$ precipitation The Mw of the TSAS may therefore be close to that of chicken serum albumin i e 65 000 (20) and at least in the range of 40,000 90 000

The two major proteins present in the active G 100 peak were transferrin and albumin as revealed by immunoelectrophoretic analysis Transferrin has been shown to enhance the DNA synthesis in spleen cell cultures (31), and to increase the proliferative response of lymphocytes to phytohaemagglutinin and antigen (30) It could therefore conceivable also stimulate the thymus in organ culture Albumin was considered necessary in tissue culture systems for certain

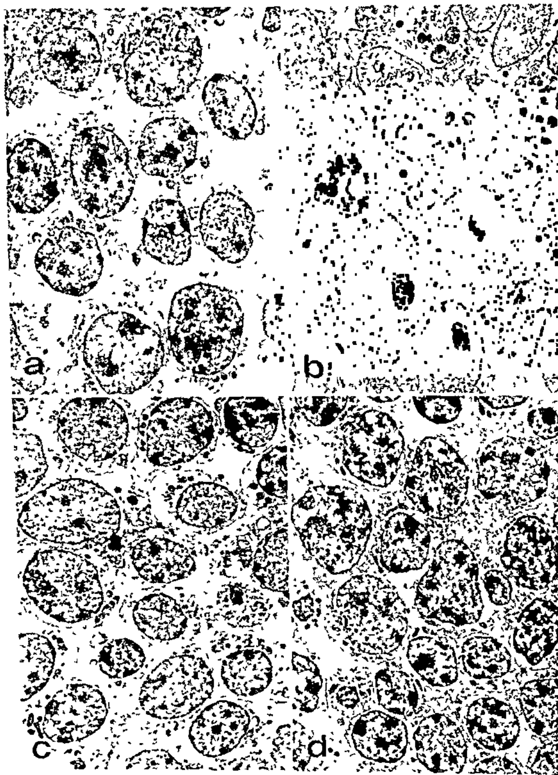


Fig 11 Ultrastructure of the same thymic anlagen as in Fig 10 a–d (5,300 ×)

types of cells (11) However, the results from fractionation of TSAS by ion exchange chromatography indicated that TSAS is not associated either with transferrin or albumin which lacked significant stimulating activity on the thymus in organ culture

Thus, the TSAS cannot merely be due to the presence of protein in the organ culture This is also evident from the fact that the serum globulins in this study or commercial ovalbumin, ovine and bovine serum albumins tested in organ culture (*J F Sällström*, unpublished results) lacked thymus stimulating activity TSAS appears instead to be a specific factor separate from the major serum proteins Significant TSAS was not detected in whole or in $(NH_4)_2SO_4$ precipitated sera from man, horse, foetal calf, rat and mouse (*J F Sällstrom*, unpublished results) This suggests that TSAS may be either species specific or peculiar to the chicken

The relative activity per mg protein of the total TSAS did not increase during the separation procedures Furthermore the apparent yield after ion exchange chromatography was only about 15 per cent The reasons for this can only partly be the losses at dialysis and ultrafiltration and major losses at other steps of the separation must therefore occur

The albumin free TSAS fraction from the DEAE Sephadex separation contained 4–5 proteins detectable by immunoelectrophoresis It is not known whether TSAS is due to one or more of these unidentified proteins This TSAS fraction supported the development of morphologically typical lymphoid cells, ultrastructurally similar to the cells obtained by whole CS

The number of lymphoid cells per anlage was dependent on the concentration of the TSAS fraction and increased successively with increasing concentration Within the tested dose range of the fraction it was not possible, however, to reach cell numbers in the anlagen of the magnitude usually obtained by 10 per cent whole CS The time dependent development of lymphoid cells in organ culture with the active DEAE Sephadex fraction was approximately the same as with whole CS during the first 3 days and then apparently reached a maximum earlier and at a lower lymphoid cell number than cultures with whole CS Whether the demonstrated differences between CS and the active fraction are simply due to low concentration of TSAS in the fraction or the lack of additional thymic growth stimulating factors in this remains to be demonstrated

Whole serum or different serum factors have been found necessary as medium supplements in most tissue or organ culture systems in various avian or mammalian species Much work has been done to isolate and characterize such growth stimulating serum factors (for review see 11, 29) Their relation to the thymus growth stimulatory factor in chicken serum remains to be determined

Further purification and characterization of the component(s) associated with TSAS is under way It also remains to demonstrate whether purified TSAS represents material that is actually essential for the growth stimulating activity of CS on the embryonic thymus in organ culture If so, the possible physiological importance of TSAS during the embryonic development of the thymus *in vivo* and for thymic lymphopoiesis in general is of great interest

This work was supported by grants from the *Swedish Cancer Society* (*511 B73 03XB*), the *Expressens Prenatalforskningsfond* and the *Faculty of Mathematics and Sciences of the University of Uppsala*

The skilful technical assistance of Mrs *Marianne* [illegible]

T Ottoson Pharmacia Uppsala Dr *F Karlsson* Dept Med Chem, Uppsala provided expert guidance during the initiation of the ion exchange chromatography experiments

I also want to thank Dr *G V Alm* for invaluable advice during this investigation and for critically reviewing the manuscript

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THE FREQUENCY AND TOPOGRAPHICAL DISTRIBUTION OF CARCINOMA IN SITU WITH BUDS IN THE UTERINE CERVIX

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The frequency and distribution of carcinoma in situ with buds (CISB) in the cervical mucosa was studied in punch and/or cone specimens from 102 consecutive cases with dysplasia or carcinoma in situ CISB was found in 20 per cent of the cases Studies of the linear extension in sections of cone specimens showed that CISB represented a small portion (20 per cent) of the total atypical epithelium and only 33 per cent of the areas with carcinoma in situ, the remaining being carcinoma in situ simplex (CISS) In 6 of the 10 cones with CISB, the lesion was limited to only 1 of the 4 quadrants Patients with dysplasia were on the average 5 4 years younger than those with carcinoma in situ No differences in age were found for cases classified as carcinoma in situ with or without buds The histological findings and age distribution indicate that CISB and CISS are histological variants of carcinoma in situ There is no evidence that CISS progresses to CISB

Based on a modification of *Hamperl's* classification (1960), carcinoma in situ of the uterine cervix has been divided into "carcinoma in situ simplex" (CISS, which is characterized by having a straight and even epithelium stroma border) and "carcinoma in situ with buds' (CISB, with irregular epithelial buds bulging into the stroma) The latter type was often found in cases of carcinoma in situ progressing to invasive carcinoma (*Rubio & Soderberg* 1969a and b) CISB was also present in specimens harbouring suspected or definite microinvasive carcinoma (*Rubio & Söderberg* 1969c and *Rubio et al* 1974) Micro-invasive carcinoma has been seen to originate at the tip of the buds in CISB (*Rubio et al* 1974)

There are no reports in the literature concerning the frequency of CISB and the linear extent of this type of lesion within the cervical mucosa The present report concerns the frequency of CISB in punch biopsies and/or cone specimens in consecutive patients undergoing conization because of dysplasia or carcinoma in situ Other parameters investigated were the linear extent of CISB in tissue sections from cone specimens and the circumferential extent of CISB in quadrants from cone specimens

Received 15 iii 74 Accepted 15 iii 74

MATERIAL AND METHODS

Cervical specimens from 102 patients have been examined All 102 patients had atypical cells in vagino cervical smears Punch biopsies from colposcopically abnormal areas and endocervical curettings were first obtained from 98 of the 102 women In the remaining 4 cases, conization was performed directly on the basis of the cytological findings



Fig 2 Carcinoma in situ simplex (× 60)

most distally located cervical gland was considered the boundary between portio vaginalis and endo cervix

The presence of lymphocytes in the stroma was recorded when present

RESULTS

a) *Histological findings* The histological diagnosis in the 102 cases is presented in Table 1 CISB (Fig 1) was found in 21 of the 102 cases (20 per cent) and dysplasia with buds was present in 11 cases (11 per cent)

Among the 98 cases from which punch biopsies were available, 55 (56 per cent) showed the same degree of atypia in biopsies and cones (cfr Table 2) In 27 cases (28 per cent) more severe changes were found in the punch biopsies than in the subsequent cone specimens The remaining 16 cones (16 per cent) contained more severe lesions than the preceding biopsies This indicates that the most severe intra-epithelial lesion had already been detected in 82 cases (84 per cent) in sections of the punch biopsies

Table 2 also shows that biopsies from the total of 30 cases of CISB or dysplasia with buds, disclosed that 17 (57 per cent) also had atypical epithelium with buds in cone specimens By comparison, only one of the 68 biopsies with CISS (Fig 2) or dysplasia without buds (i e 1 5 per cent), had buds in atypical epithelium in cone specimens

Moderate to heavy lymphocytic infiltration was present in the stroma in 6 of the 21 cases (29 per cent) of CISB and in 10 of the 37 cases of CISS (26 per cent)

The Circular Distribution of Carcinoma in Situ in Cone Specimens

CISB was limited to only one quadrant in 6 of 10 patients and CISS to one quadrant in 17 of 36 patients (Table 3) No major differences were found in the number of quadrants involved by dysplasia with or without buds in cone specimens It also appears that carcinoma in situ or dysplasias with or without buds do not have a predilective loca-

TABLE 3 *The Distribution of Carcinoma in Situ with Epithelial Buds (B) and with Straight Epithelial-Stroma Border (S) in 46 Cone Specimens*

CISB present in	No cones CISB	Histology in the remaining quadrants		
		CISS	Dysplasia B	Dysplasia S
1 quadrant	6	4	3	3
2 quadrants	3	1	–	–
3 quadrants	1	1	1	–
4 quadrants	–		–	–
Total	10	6	4	3

CISS present in	No cones CISS	Histology in the remaining quadrants	
		Dysplasia B	Dysplasia S
1 quadrant	17	4	11
2 quadrants	5	2	4
3 quadrants	9	–	2
4 quadrants	5	–	–
Total	36	6	17

TABLE 4 *The Distribution of Carcinoma in Situ and Dysplasias in the Various Quadrants of the Cone Specimens in 10 Cases of CISB*

Cone no	Histology in each quadrant			
	I	II	III	IV
1	GDB	CISB	GDB	GDB
2	CISB	GDB	GDS	CISS
3	CISS	CISB	CISS	CISS
4	CISB	N	N	N
5	CISS	N	CISS	CISB
6	CISS	CISB	N	GDB
7	CISB	N	N	CISB
8	CISS	CISB	CISB	CISB
9	GDS	CISB	CISB	MDB
10	CISB	CISB	CISS	MDS

GD denotes grave dysplasia MD moderate dysplasia S straight epithelium-stroma border and B *epithelial buds*

lization in any particular quadrant of the cone specimen

The distribution of dysplasia and carcinoma in situ in the various quadrants in 10 cone specimens classified as CISB is presented in Table 4 The great variation in the circular distribution of dysplasias and carcinoma in situ with or without buds is obvious Of particular interest is case number 3 where only one quadrant contained CISB, whereas the other 3 quadrants contained CISS

The Linear Distribution of Carcinoma in Situ in Tissue Sections of Cone Specimens

The linear distribution of carcinoma in situ and dysplasia in the sections of 10 cases (5 with CISB and 5 with CISS) is shown in Table 5 It is seen that CISB had a more extensive distribution in the endocervix than in the portio vaginalis (in one of the cases CISB was localized exclusively to the portio vaginalis) No case of CISB was limited exclusively to the endocervix This pattern, however, was found in 2 cases of CISS

CISB represented on the average 20 per cent of the total atypical epithelium and 33 per cent of the areas with carcinoma in situ (Table 5)

Irregular buds protruding into the stroma were also encountered in areas with normal squamous epithelium (Fig 3) in 23 of the 37 cases (62 per cent) of CISB or dysplasia with buds Normal epithelium with buds was also found in 17 of the 65 cases of CISS or

continued

were found to be interposed in the normal epithelium without buds Its presence could not be correlated to any particular phase of the ovalutory cycle as deduced by the cyclic state of the mucosa of the corpus uteri in the various cases

For these reasons the non committal name "carcinoma in situ simplex" is suggested rather than the term proposed by *Hamperl* "carcinoma in situ with simple replacing growth" The descriptive term carcinoma in situ with buds was preferred to the one pro posed by *Hamperl* (i e with bulky outgrowth) since the growth properties of such structure has not been fully investigated

The finding that most of the cases (i e 95 per cent) of CISB and dysplasia with buds *in cone specimens also presented similar* lesions in the preceding biopsies but that only 50 per cent of the cases of CISB or dysplasia with buds in punch biopsies presented similar lesions in cone specimens suggest that the areas with CISB or dysplasia with buds may be preferentially selected for biopsy because some subtle difference in their colposcopic appearance may influence the gynaecologist

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I am indebted to professor *B Lagerlof* and dr *C Grant* for interest in and constructive critisism of this investigation

The investigation was supported by a grant from the *Swedish Cancer Society*

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ENZYME HISTOCHEMICAL INVESTIGATIONS OF HETEROTOPIC GASTRIC EPITHELIUM IN THE DUODENUM

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Heterotopic gastric epithelium in the duodenum was investigated by the following enzyme histochemical reactions NADH tetrazolium reductase lactic acid dehydrogenase succinic acid dehydrogenase glucose 6 phosphate dehydrogenase alkaline phosphatase acid phosphatase aminopeptidase and non specific esterase The enzyme activities were compared with that of normal fundic mucosa No differences could be disclosed

Heterotopic gastric epithelium has been demonstrated in the duodenum (*James* 1965 *Hoedemacker* 1968 *Johansen* 1972 *Suzuki et al* 1972) The epithelium may sometimes form macroscopically demonstrable polyp like lesions (*Johansen & Hansen* 1973) It has been shown that the heterotopic epithelium is more often seen in patients with a high acid secretion (*Johansen & Hansen* 1973)

The heterotopic cells including surface epithelial cells parietal cells and chief cells are all stained like the homotopic cells using ordinary simple staining methods or the PAS reaction It is the purpose of this paper to investigate the activities of the heterotopic cells with a series of enzyme histochemical methods and to compare the results with those of the homotopic cells

Received 18 74 Accepted 15 1 74

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MATERIAL AND METHODS

Mucosal specimens from the fundic part and from the first part of the duodenum were obtained from 44 gastric resection specimens received immediately after the operation 11 cases proved to have heterotopic epithelium in the duodenum and in one case this was macroscopically demonstrable The reasons for resection were duodenal ulcer in two cases prepyloric ulcer in four cases gastric ulcer in three cases and carcinoma in one case In the last case resection was performed on account of the polyp like metaplastic areas

The following enzyme histochemical reactions were carried out

Dehydrogenases NADH tetrazolium reductase (NADH Tr) lactic acid dehydrogenase (LDH), succinic acid dehydrogenase (SDH) and glucose 6 phosphate dehydrogenase (G 6 DH) The reactions were carried out on fresh frozen 8 μm thick cryostate sections The method indicated by *Thomas & Pearse* (1961) was used The tissue was incubated at 37 C for 20 and 30 minutes No post fixation was used Control sections incubated in media containing no substrate were run with all procedures

Hydrolytic enzymes alkaline phosphatase was demonstrated by the method of *Pearse* (1960) In a few cases the method of *Burstone* (1958) was also used Acid phosphatase was performed as described

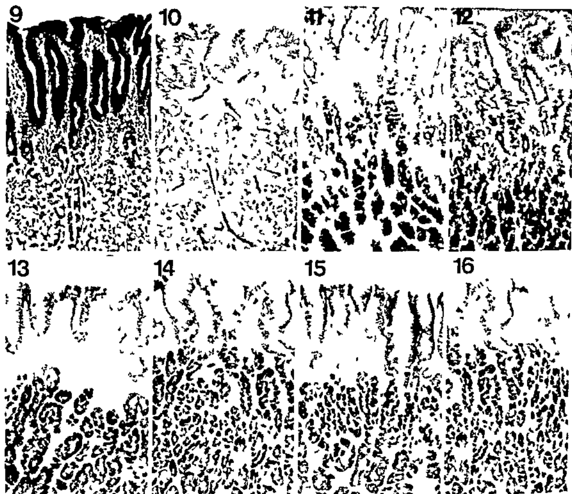


Fig 9–16 Enzyme histochemical patterns of the fundic mucosa × 100

Fig 9 PAS reaction

Fig 10 Alkaline phosphatase only activity in the capillaries

Fig 11 Acid phosphatase the activity of the chief cells predominates, slight activity in the parietal cells and only trace quantities in the surface epithelium

Fig 12 Non specific esterase as acid phosphate (see text)

Fig 13 NADH Tr the parietal cells are strongly positive, the chief cells and surface ep thel al cells d scl se a sl ght activity

Fig 14 LDH same activity as NADH Tr but a little slighter

Fig 15 SDH as LDH

Fig 16 G 6 DH as LDH

the aminopeptidase reactions were applied *The dehydrogenase* reactions were slightly positive in all es but the activity was weaker than in the arietal cells

In no case differ ces between the hetero topic cells and the milar cells localized in the stomach were fo nd (Fig 9 16)

DISCUSSION

Heterotopic gastric epithelium in the duo denum has never been enzyme histochemical ly investigated before The present work has demonstrated that the activity of the cells whether localized in the duodenum or in the

fundic part of the stomach, is the same

The results of the reactions applied to the cells in their normal position are in close agreement with previous investigations (*Villareal* & *Burgas* 1955, *Wattenberg* 1959, *Planteydt* & *Willighagen* 1960, *Niemi et al* 1961, *Plosscowe et al* 1963, *Ragins et al* 1964, *Ragins* & *Dittbrenner* 1965) The slight reaction of the parietal cells and the surface epithelium with the non specific esterase reaction has also been noticed by *Gomori* using the alpha naphthyl acetate method (*Pearse* 1960)

The consequence of the heterotopia—if any —can only be suggested The occurrence of small areas of gastric surface epithelium with a secretory ability—judged by the staining reactions—in a mainly absorbtive region may interfere with the resistance of the surface

The strength of the dehydrogenase activity in the parietal cells is supposed to be an ex pression of the cell function (*Burholt* & *Myren* 1968, *Myren* 1971) The strong activity in the heterotopic parietal cells seems to indicate that acid secretion takes place When the cells are found in considerable numbers they may influence the pathogenesis of duodenal ulcer The common occurrence of the phenomenon in duodenal ulcer patients supports this view (*Suzuki et al* 1972, *Johansen* & *Hansen* 1973)

Efficient technical assistance by Mrs *Sanne Holmberg* is gratefully acknowledged

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The bone marrow was removed by a sharp blast of air and the bone was rinsed in saline The distal portion of the left tibia was used for whole bone preparation and the identical part of the right tibia for micro dissection in the experimental group

The distal part of the left tibia was defatted and dehydrated through acetone and was finally pulverized in a Wiley Laboratory Mill provided with a 60-meish sieve

The distal part of the right tibia was frozen in propane immersed in liquid nitrogen The specimens were then stored at −20° C until sectioned 50 μ thick sections were cut using a sliding microtome (Jung, K, AG, Heidelberg) in a cryostat at −20° C They were transferred to tape (Scotch Magic Transparent tape No 810) The microdissection was carried out in a binocular dissection microscope at a magnification of 25 × The parts of a section not exactly identifiable were discareded Three areas were chosen for analyses as shown in Fig 1

Area A represents the area of most active periosteal proliferation in the tibial diaphyses All this bone was formed during the period of fluoride supplement (Fig 2)

Area B consisted mainly of bone formed during the period of fluoride supplement but had a less trabecular appearance than the bone of area A A small part of this bone could have been formed before the fluoride supplement (Fig 3)

Area C is an area localized opposite to A and B and showed no signs of increased bone format on The bone in area C had to a greater extent formed before the fluoride supplement than that in area B (Fig 4)

As the ash content of the tape was less than 0 2 per cent and as it did not contain measurable amounts of the minerals analysed, no attempt was made to remove the tape from the bone sections

Roughly 40 mg of each bone sample was ashed in a platinum crucible at 700° C overnight

Analytical Methods

Chemical analyses were done on whole bone powder in the control group and on whole bone powder and microdissected bone samples from each animal in the experimental group

Fluoride determination was carried out on about 10 mg of ash as described by *Rosenquist* (1973 a) us ng an ion selective electrode The ash was dissolved in 1 0 ml of 1 0 M HCl and 3 ml of 1 9 M acetate buffer (pH 5 5) and 5 ml of water was added

Phosphate determination was performed on 0 1 ml of the sample solutions used for the fluoride determinations using the method of *Fiske & Subbarow* (1925) as described by *Augustinsson* (1966) To the standards and blanks were added the same amounts of HCl and acetate buffer as to the unknown samples

Calcium and magnesium determinations were done on 10 mg of ash by atomic absorption spectrophotometry, using a Unicam SP 90 spectrophotometer (Unicam Instr, London England) SrCl solution was used to suppress the phosphorus interference with the calcium determination at a final SrCl concentration of 1200 p p m (*Willis* 1960)

The values reported are averages of duplicate determinations

Statistics

The degree of significance was tested by Student s t test

RESULTS

Four animals died of infection, one in the control group and three in the experimental group All other animals remained healthy and the weight gain was uniform in the two groups

Chemical Analyses

The concentrations of fluoride, calcium magnesium and phosphorus in the bone ash are given in the Table 1 The fluoride concentration was higher in bone from areas A and B than in the 'whole bone' and the bone from are C, respectively, ($p<0\,001$) It was slightly higher in area A than in area B ($p<0\,05$) The whole bone concentration of magnesium was higher in the experimental animals than in the controls ($p<0\,01$) but no difference between the different areas was found Calcium and phosphorus concentrations and Ca/P ratios were the same in all areas analysed and without difference between normal and treated animals

DISCUSSION

The three areas chosen for microdissection represented morphologically different types of bone from the middiaphysis of the tibia Area A consisted solely of newly formed irregular trabecular bone, entirely formed during the period of fluoride supplement As expected the fluoride concentration was highest in this area Also in area B, the fluoride con

TABLE 1 *The Concentrations of Calcium, Magnesium, Fluoride and Phosphorus in per cent of Bone Ash of Tibia from Normal Rabbits and Rabbits Given Fluoride during 14 Weeks*

Mineral	Whole bone average		Microdissected		
	Normal	Experimental	Experimental bone areas A	B	C
	n 9	7	7	6	6
F	0 02±0 003	0 83±0 12	1 28±0 08	1 14±0 11	0 85±0 10
Ca	37 2±2 1	37 8±3 3	39 3±2 0	37 5±2 4	37 1±1 8
Mg	0 66±0 04	0 76±0 08	0 87±0 13	0 77±0 10	0 80±0 11
P	19 0±0 6	17 8±1 2	19 2±1 6	19 6±1 3	19 3±1 1
Ca/P	*1 9±0 1*	*2 1±0 1*	*2 0±0 2*	*1 9±0 1*	*1 9±0 1*

Values for 'whole bone and microdissected bon bone areas according to Figure 1 M±SD

centration was high as the bone in this area was mainly formed during the fluoride supplement The morphology was, however, more regular and the bone contained fully developed osteones In area C, the bone structure was normal and the fluoride concentration approximated that of the whole bone average The fluorotic bone (area A and B) constituted only a small part of the "whole bone" sample as the exostoses were limited to the lateral part of the middiaphysis of the tibia, *viz* the proximal part of the specimens analyses This explains why the fluoride concentration in the "whole bone" was not found to differ from the concentration in the bone of area C The share of bone in area C formed before the period of fluoride supplement constituted roughly 30 per cent of the total as estimated by fluorescence microscopy on fluorochrome labelled specimens from identical parts of the tibia (*Rosenquist* 1973b) From the values on the fluoride concentration of area A, the fluoride concentration in area C could be calculated to about 0 9 per cent This figure corresponds well with the figure obtained—0 85 per cent Remodelling and/or ionic exchange could also have influenced the retention of fluoride in the "prefluorotic" bone Thus the fluoride concentration in the bone formed during the period of fluoride supplement in the different areas seemed to be of the same magnitude Yet only the osteogenic cells of the lateral part of the middiaphysis of the tibia laid down excessive amounts of periosteal new bone forming fluorotic exostoses The sites of these exostoses were normally not the sites of more active bone formation than was area C (*Rosenquist* 1973b) Neither were any muscle or tendon insertions localized to this area One wonders whether there might be any difference in fluoride sensitivity of the osteogenic cells in the two areas or whether differences in mechanical stress may make the lateral part more sensitive to stimulatory agents such as fluoride

With respect to the other mineral components, the only difference between normal and experimental bone was the increased concentration of magnesium after fluoride ingestion This has also been found in "whole bone" investigations (*i e McCann & Bullock* 1957, *Zipkin et al* 1960) and in studies of fluorotic exostoses (*Weidmann et al* 1959) However, in the experimental bone there was no direct relationship between the magnesium and fluoride concentrations It is possible that the increased magnesium concentration in the experimental bone merely reflects the increased metabolic activity, and is not due to formation of any magnesium fluoride compound

Financial support was given by the *Swedish Medical Research Council* (project 24 X 3925)

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TABLE 1 *Number of Guinea Pigs in Each of the 6 Groups with Avillous Lesions in the Duodenum and Gastric Erosions*

Group number	Degr carr	Fasting	Days	Avillous lesions in the duodenum	Gastric erosions
I	+	+	2	3	0
II	+	+	4	2	3
III	+	—	2	1	1
IV	+	—	4	0	4
V	—	+	2	7	1
VI	—	+	4	7	1

Planimetric studies on photographs were carried out on all animals with avillous lesions

RESULTS

Avillous Areas

As demonstrated previously (12) fasting induces avillous areas in the proximal part of the duodenum This phenomenon was observed in all guinea pigs of groups V and VI regardless of the duration of the experiment

Among the 14 guinea pigs of groups I and II which had received d c during the fasting, 4 exhibited avillous areas

Among the 14 guinea pigs of groups III and IV which had received d c without being fasted one had a few avillous patches in the duodenum

The difference in the frequency of avillous patches in the duodenum between groups I + II and V + VI is statistically significant (Fisher s exact test 2 $p < 0.002$)

The average area of avillous lesions in group I and II was 13 per cent of the total area of the proximal 6 cm of the duodenum (5 guinea pigs) The average for group V and VI was 35 per cent (14 guinea pigs) The difference is however not statistically significant

Superficial Gastric Erosions

Among the 14 guinea pigs of groups V and VI which had not received d c 2 (14 per cent) had erosions in the stomach Among the 28 guinea pigs of groups I IV which had received d c for 2 or 4 days 8 (29 per cent) had erosions in the stomach The difference is not significant Among the 14 guinea pigs of groups II and IV which had received d c for 4 days 7 (50 per cent) exhibited erosions However, there is not a statistically significant difference between groups V and VI in relation to II and IV

DISCUSSION

D c has often been used experimentally and also clinically since 1959 when *Anderson et al* (1) demonstrated that like the chemically related heparin (10) it caused local inhibition of the proteolytic activity of pepsin and that on oral administration to guinea pigs it could inhibit the development of histamine induced ulcerations in the duodenum

Later it was found that d c exerted an effect upon acid production presumably by liberating histaminase (3) This was first demonstrated after oral administration (2), later also after parenteral administration (13)

The clinical effect of d c in patients with gastro duodenal ulcers has been investigated by several authors (4, 7)

Eagleton et al (5) measured the inhibitory effect of subcutaneously injected d c upon the fasting acid production in guinea pigs 18 hours after subcutaneous injection of 400 mg/kg they found the volume of gastric juice reduced to less than one third and the acidity by more than 10 per cent in relation to normal fasting values (17) Moreover, there was a significant reduction of the volume of the

gastric juice as well as of total acid concentration following maximum stimulation during the period 6–24 hours after the injection The values did not return to normal until the 3rd day

In a dose response study on d c Eagleton et al found that 400 mg/kg administered subcutaneously is the minimum dose to cause maximum depression of acid production (6) At this dosage the maximally stimulated acid production was lesser than the normal fasting acid production

In the present study the initial dose was 400 mg/kg This dose was administered 6 hours before the experiment was started Thus, at the time when the experiment was started, and some of the guinea-pigs were to fast, acid production was sufficiently inhibited As stated above, the effect of d c is at a maximum between 6 and 24 hours after the administration

As a maintenance dose the guinea pigs received one third of the initial dose at 18 hour intervals Experiments in which higher doses were given were followed by 4 deaths among 14 guinea pigs, possibly because of an accumulation of d c

As stated, d c inhibits acid production In the present study it was demonstrated that d c significantly inhibits the development of avillous areas in the duodenum during fasting This further supports the previously advanced hypothesis that the formation of these areas is conditioned by the effect of acid gastric juice in the duodenum Other factors which point into the same direction are that guinea-pigs are known to have a high fasting acid production (14), that the changes are most pronounced proximally in the duodenum, gradually decreasing distally, that the desquamation of the villi sets in after merely 12 hours' fasting i e before a deficiency of important nutrients might manifest itself, and that avillous areas may be encountered in the duodenum of patients with a high fasting acid production (duodenal ulcer and Zollinger Ellison syndrome)

Administration of d c apparently increased the tendency to develop superficial erosions in the stomach, in the guinea pigs that were fasted as well as in those that were not fasted However, the difference between the groups was not statistically significant Fasting *per se* may be followed by the development of erosions in the stomach (12) In the present experiments erosions were found in 2 out of 14 guinea-pigs which had only been fasted The cause of this possible effect of d c is unknown It is known that orally administered degraded carrageenan in guinea pigs may lead to the development of an appearance suggesting ulcerative colitis in the course of less than a month (15) A toxic effect is also conceivable (since 4 out of 14 guinea-pigs died when it was attempted to use a higher maintenance dose)

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dal carbon and in one rat which did not receive injections The fixative used was a 4 per cent glutaraldehyde containing either 1) 3 per cent dextran in 0 02 M cacodylate buffer adjusted to pH 7 4 or 2) 2 per cent dextran, 0 3 per cent NaCl in 1 6 per cent phosphate buffer adjusted to pH 7 4

The perfusion was performed retrogradely through a catheter inserted into the common carotic artery A very loose ligature placed around the portal vein at the start of the operation made it possible to cut the portal vein at the same moment as the perfusion was started Perfusion time was 15 to 20 minutes with 30 to 40 drops/minute

Immersion fixation in vivo was carried out in the remaining three rats using a 6 per cent solution of glutaraldehyde in the same buffers as mentioned above The abdominal window was removed and the small intestine was very gently withdrawn from the abdominal cavity and placed in a bath of the fixative until the circulation in the blood vessels stopped The small intestine was then cut off and placed in fixative for 12 hours

After fixation twenty arterioles from the upper half of the jejunum were dissected out under the stereo microscope Fifteen of the arterioles originated from the five rats injected with saline and colloidal carbon, the remaining five arterioles from the non treated rats Measured in vivo, the diameter of the arterioles was 60–100 microns The arterioles fixed with phosphate buffered glutaraldehyde were post fixed for one hour in two per cent osmium tetroxide buffered with phosphate, those fixed with cacodylate buffered glutaraldehyde were post fixed for one hour in two per cent osmium tetroxide buffered with veronal acetate at pH 4 8 to 5 0 and block stained with 0 5 per cent uranyl acetate in veronal acetate buffer Finally, the arterioles were dehydrated and embedded in Epon For light microscopy, one micron thick sections were cut with an ordinary microtome and stained with toluidine blue For electron microscopy sections were cut at 800–1000 Å with an ultramicrotome stained with uranyl acetate and lead citrate and studied under a Hitachi HS 7 S electron microscope

RESULTS

The five arterioles from the two rats which were fixed immediately after anaesthesia showed the same ultrastructural morphology as the arterioles from the rats injected intravenously with physiological saline and colloidal carbon particles

The endothelium was continuous without fenestrations or intercellular gaps, measuring 0 25–2 5 microns in thickness In cross sections of the arterioles, 10–40 endothelial cells were observed connected by distinct junctions measuring about 160 Å in width In most cases, these were easily recognized as gap junctions (Figs 3 a d), but probably also tight junctions (*Brightman & Reese* 1969) exist (Fig 4)

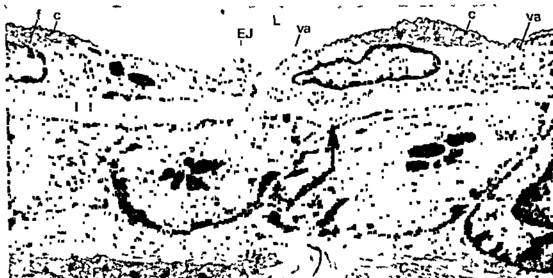
Interdigitations between neighbouring endothelial cells were often observed (Figs 1, 3a, 7), one or both cells forming a protrusion into the lumen of the arteriole (Figs 1, 2, 7, 10)

From the abluminal surface of the endothelial cells protrusions penetrate the internal elastic lamina (Figs 2, 8, 10), sometimes establishing junctions with the smooth muscle cells of media (Fig 5) The endothelial junctions were often situated corresponding to these protrusions (Figs 2, 10)

The nuclei of the endothelial cells were oval or flattened with a smooth or slightly indented surface (Figs 1, 8, 10)

Fig 1 Slightly oblique section through part of an arteriolar wall The endothelial cells (E) are connected by distinct junctions (EJ) The cell surface is smooth, the endothelial nucleus (ne) only slightly indented Many vesicles (ve) and caveolae (c) are seen in the cytoplasm The internal elastic lamina (IEL) is fenestrated and in the fenestra an endothelial protrusion (arrow) is seen Media consists of one or two layers of smooth muscle cells (SM) In adventitia (A), some collagenous fibrils (C) and a small part of a fibrocyte (F) are seen L lumen, f filaments m mitochondria va vacuoles Perfusion fixation Cacodylate buffered glutaraldehyde Uranyl block stained × 10 600

Fig 2 An endothelial junction (EJ) is situated corresponding to an endothelial protrusion (EP) in a fenestra (FEN) of the internal elastic lamina (IEL), which in this picture is nearly without any electron density The endothelial protrusion is separated from the smooth muscle cell of media (SM) by basement membrane material (BM) Corresponding to the endothelial junction also endothelial protrusions into the lumen (EPL) are seen The adventitial basement membrane of media (ABM) is well defined and thick ab attachment bodies db dense bodies, er endoplasmic reticulum *f filaments g glycogen, m mitochondria* mvb multivesicular body, ne nucleus of endothelial cell Perfusion fixation Phosphate buffered glutaraldehyde × 42,500



CARBON
EP
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2

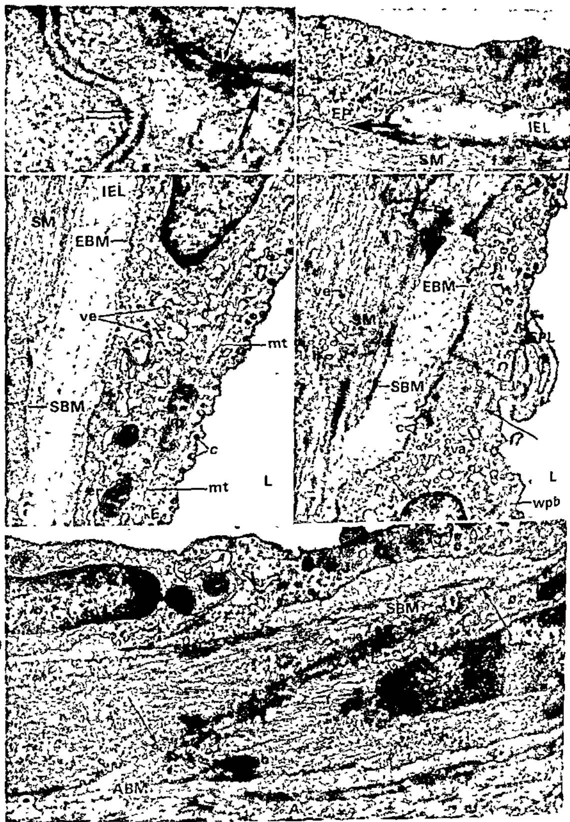

E
EP
IEL
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ve
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c
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E
ve
EBM
SM
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c
va
L
wpb
SBM
ABM
A

Fig 4 Detail from another endothelial junction showing what might possibly be a tight junction The total width of the two cellular membranes facing the point of the arrow is about 120 Å, which is less than twice that of the unit membrane (thick arrow) The usual interspace (double arrow) is about 100–200 Å Perfusion fixation Cacodylate-buffered glutaraldehyde Uranyl block stained × 125,000

Fig 5 Myoendothelial junction (arrow) The two cells are separated by a space measuring 100–150 Å E endothelial cell, EP endothelial protrusion, IEL internal elastic lamina, SM smooth muscle cell, f filaments Perfusion fixation Phosphate buffered glutaraldehyde × 36,000

Fig 6 Part of an endothelial cell and the under lying internal elastic lamina. In the endothelial cell (E), thick and thin filaments (f) are clearly seen Microtubules (mt), many caveolae (c), vesicles (ve) and a small amount of endoplasmic reticulum (er) are also observed The internal elastic lamina (IEL) appears amorphous with small more electron dense spots The basement membrane of the endothelial cell (EBM) and that (SBM) of the smooth muscle cell (SM) are well seen, the latter being a little thicker and a little more distinct than the former m mitochondria Perfusion fixation Cacodylate buffered glutaraldehyde Uranyl block stained × 28,000

Fig 7 An interdigitated junction (EJ) of the endothelium A fingerlike protrusion from one endothelial cell into another is transversely cut (arrow) In the cytoplasm of the endothelial cell, many caveolae (c), vesicles (ve) and vacuoles (va) are seen as well as a Weibel Palade body (wpb) In the visible part of the cytoplasm of the smooth muscle cell of the media (SM), filaments (f), vesicles (ve), caveolae (c) and a small amount of rough endoplasmic reticulum are seen EBM endothelial basement membrane, EPL endothelial protrusion into lumen (L), SBM basement membrane of the smooth muscle cell Perfusion fixation Cacodylate buffered glutaraldehyde Uranyl block stained × 28,000

Fig 8 Part of arteriolar wall showing junctions between smooth muscle cells of media (arrows) A adventitia, ABM adventitial basement membrane, EJ endothelial junction, EPL luminal endothelial protrusion, FEN fenestra in the internal elastic lamina, SBM basement membrane of the smooth muscle cells ab attachment bodies, c caveolae, er endoplasmic reticulum, f filaments, m mitochondria Perfusion fixation Phosphate buffered glutaraldehyde × 38,000

luminally and abluminally situated muscle cells—continued in the basement membrane facing the internal elastic lamina and that facing the adventitia Between the smooth muscles cells the basement membrane was thin—sometimes invisible, in other places measuring 500–800 Å—but at the adventitial side of media it was continuous, well-defined and measured 600–900 (1000) Å in thickness (Figs 1, 2, 3a, 8, 10)

Besides the basement membranes some thin collagen fibrils were observed intercellularly (Fig 3a) In a few cases, junctions were found between the smooth muscle cells, which were then separated by a space measuring about 100 Å (Fig 8)

The smooth muscle cells contained a nucleus, which measured 20 25 microns in length and 0 5-1 0 micron in width The myofilaments were thin, measuring 50 70 Å in thickness and in only a very few cases thick, measuring 100 Å Between the myofilaments big mitochondria, rough endoplasmic reticulum, about 2000 Å large lipid vacuoles, a well developed Golgi apparatus, small vacuoles and glycogen granules were observed (Figs 1, 2, 3a, 7, 8, 10)

On the surface of the smooth muscle cells, caveolae, and inside the cells, vesicles, measuring 400–600 Å in diameter, were seen in varying number (Figs 1, 2, 3a, 8, 10)

Adventitia was formed by loose connective tissue with bundles of collagen fibrils, fibrocytes, macrophages, eosinophilic granulocytes, plasma cells and nerves (Figs 1, 10)

Colloidal carbon particles were situated in the lumen of the arterioles as single black homogeneous round particles measuring from 200 Å in diameter or 2000–3000 Å conglomerates of particles (Fig 2) There was no sign of passive or intercellular passage of carbon through the endothelial layer Neither was there any sign of active or transcellular transport of carbon particles, except in one section, which showed carbon particles placed in a membrane-limited body measuring 3000 Å in diameter and situated luminally in an endothelial cell


EP
IEL
SM
mt
L
E
va
L
wpb
L

DISCUSSION

In the present study of the ultrastructure of normal submucosal intestinal arterioles in rats, the structure was similar in rats fixed immediately after anaesthesia and in rats, which had been anaesthetized for three hours, injected intravenously with small amounts of physiological saline and finally injected with colloidal carbon particles It could thus be concluded that these experimental procedures did not disturb the normal arteriolar architecture

Descriptions of the normal arteriolar structure in general and in different animals and organs, and of the components in the normal vascular wall are given in several publications (for literature see *Majno* 1965, *Rhodin* 1967, *Bloom* & *Fawcett* 1968, *French* 1970) Mesenteric arteries are described by *Ooneda et al* (1965) and by *Mattheus* & *Gardner* (1966), but apart from a short description by *Goldby* & *Beilin* (1972), no study of the ultrastructure of the normal intestinal submucosal arterioles in the rat has been found

The ultrastructure of the normal submucosal arterioles from the upper half part of the jejunum did not differ in essentials from the above mentioned descriptions

The internal elastic lamina is a fenestrated membrane, in only a few studies is the myoendothelial junction through some of the fenestrae mentioned (*Moore* & *Ruska* 1957, *Fawcett* 1959 *Mattheus* & *Gardner* 1966, *Rhodin* 1967, *Goldby* & *Beilin* 1972) *Rhodin* (1967) in a study of arterioles from muscle fascia found myoendothelial junctions only in arterioles with a diameter of less than 50 microns, but in the present work as in that of *Goldby* & *Beilin* (1972), they were seen in arterioles with a diameter ranging from 60 to 100 microns An additional finding in the present work was the rather frequent localization of interendothelial junctions in the endothelial protrusions

Karnovsky (1967) found passive (intercellular) movement of peroxidase (40 Å particles) through the endothelium in muscle capillaries Active (vesicular) transfer of tracer particles with a size ranging from 40 to 300 Å is described in many capillaries (*Jennings et al* 1962, *Simionescu et al* 1972) and recently for some small cerebral arterioles (*Westergaard* & *Brightman* 1973) Endothelial junctions of "tight" and "gap" type do not allow passive movement of colloidal carbon particles (200 Å or more) Active (vesicular) transfer could be expected to take place (*Simionescu et al* 1972, *Westergaard* & *Brightman* 1973), but was not found in the present study

Our conclusion is that colloidal carbon circulating for 15 minutes does not penetrate the endothelium in normal intestinal submucosal arterioles with a size ranging from 60–100 Å

Fig 9 Tangentially cut section of endothelial cell facing a big fenestra in the internal elastic lamina The irregular light areas in the right of the picture are parts of the arteriolar lumen (L) Several organelles are seen EBM endothelial basement membrane, SBM basement membrane of smooth muscle cells SM smooth muscle cells c caveolae cen centrosphere region f filaments go Golgi complex, m mitochondria mt microtubules, ve vesicles Perfusion fixation Cacodylate buffered glutaraldehyde Uranyl block stained × 28,500

Fig 10 Part of one of the smaller arterioles An endothelial junction (EJ) is seen corresponding to the endothelial protrusion (EP) in the fenestra of the internal elastic lamina (IEL) A adventitia, ABM adventitial basement membrane, SBM basement membrane of smooth muscle cells, c caveolae, en endothelial nucleus, f filaments m mitochondria Perfusion fixation Phosphate buffered glutaraldehyde × 26,000

The electron microscope used in the present study was made available by a grant (A 1/65) to Dr *H Moe* from the *Danish Medical Reseach Council* and the study was further supported by a grant to Dr *J Bing* from *King Christian X Foundation*

The authors are very grateful to Dr *Harald Moe* and Dr *Jens Bing* for reading and criticizing the manuscript We wish to thank Dr *Jørgen Rostgaard* for helpful discussions and Miss *Kirsten Sjøberg* for technical assistance

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FOCAL AORTIC INJURY CAUSED BY CANNULATION: INCREASED PLASMA PROTEIN ACCUMULATION AND THROMBOSIS

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Previous studies have shown evidence of 'spontaneous' injury associated with increased permeability in focal areas of non atherosclerotic animal aortas In this study, a mild intimal injury was induced by brief cannulation of the thoracic aorta of rabbits and pigs to examine whether focal aortic plasma accumulation is due to the injury per se or to other effects Evans blue, a marker of plasma albumin, was used to visualize plasma accumulation The animals were killed 10 minutes to 5 hours after cannulation In all animals, blue-stained, sharply demarcated streaks were found in the thoracic aorta By microscopy, the streaks showed loss of endothelium and frequently, platelet thrombi with or without fibrin, leucocytes, or red blood cells The basal platelets were in contact with exposed basement membrane, or with fibres of elastin or collagen The inner layers of the aortic wall corresponding to the streaks showed signs of oedema In contrast, unstained areas of the aorta had an intact endothelium, and neither thrombosis nor oedema was present The observations show that a mild and brief injury of the aortic intima can cause both mural thrombosis and leakage of plasma into the inner layers of the aortic wall The extent of the leakage appeared to be independent of haemodynamic effects The injury was similar to, but more severe, than most spontaneous lesions

In previous studies we observed a correlation between focally increased accumulation of plasma protein in non atherosclerotic aortas of rabbits and pigs and focal intimal injury with platelet accumulation and thrombosis (11, 16) We concluded that the most likely explanation of the increased permeability for plasma protein was the intimal injury (11) The cause of the intimal injury could be haemodynamic factors alone (7) or platelet interaction with the aortic wall, governed by haemodynamic factors (10, 11)

This theory presupposes that a mild injury of the aorta may cause increased permeability to plasma protein Alternative explanations of the focal protein accumulation could be 1) decreased clearance of proteins seeping into the vessel wall 2) increased plasma leakage from the vasa vasorum (1, 18), or 3) the

Received 1 i 74 Accepted 1 v 74

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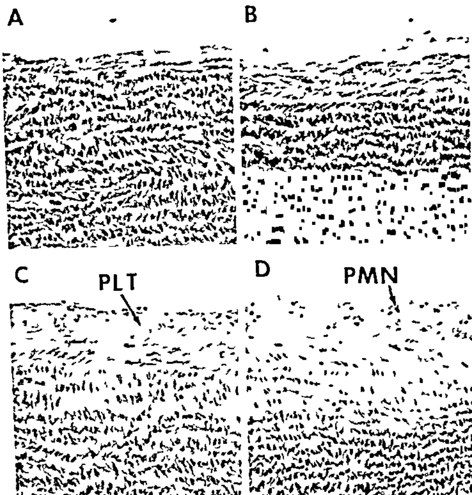


Fig 2 A sequence of micrographs of a cross section of a blue streak from the aorta of a rabbit after Evans blue infusion and cannulation The pictures show the lesion from the periphery (A) to the centre (D) Notice from A through D an increasing height of the platelet thrombus (PLT) covering the vessel surface and an increasing oedema of the innermost layers of the vessel wall The platelet masses appear either as a fairly uniform layer or as columns radiating into the lumen In C and D a few leucocytes (PMN) are intermingled with the platelets Haematoxylin phloxin saffron × 200

rabbits one sample for each examination method was taken from a blue streak and an unstained area in the pigs 3 to 5 samples for each method were collected from the blue streaks and 1 to 3 samples from the unstained areas

The preparation methods for light and electron microscopy were as previously described (16)

RESULTS

Gross examination showed sharply demarcated [illegible] stain on the intimal surface in parts of the aorta which were reached by the cannula (Fig 1) No other blue staining was observed in animals killed within an hour of Evans blue infusion The results were similar both in rabbits and pigs and in animals given Evans blue either before or after the cannulation No deposit on the streaks was visible with the naked eye

Light microscopy of sections from unstained areas in both rabbit and pig aortas showed an intact endothelium resting on an uninter

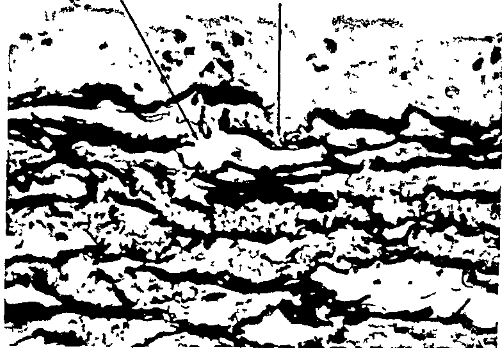


Fig 3 A blue streak in the aorta of a rabbit after Evans blue infusion and cannulation The platelet thrombus covers a vessel wall devoid of endothelial cells and with ruptured superficial elastic fibres (arrows) Verhoeff's elastic stain, × 810

rupted internal elastic membrane or a thin intima of smooth muscle cells and thin elastic and collagen fibres The texture of the aortic wall was uniform throughout all layers

In animals of both species surviving for 10 minutes or less, sections from the aorta corresponding to the blue streaks invariably revealed loss of endothelial cells (Fig 2) The injured areas were sharply demarcated In some areas the entire endothelial layer was lacking, in other areas one or a few endothelial cells persisted here and there Sheets of apparently detached endothelial cells could be observed near the intimal surface The elastic laminae near the intima were frequently ruptured (Fig 3)

The inner layers of the aortic wall underneath the intimal lesions usually showed an increased space between the tissue components (Fig 2 The loosening of the texture was most pronounced in the middle of the cross section of the injured area and tapered off towards the periphery Fig 2) Leucocytes had not migrated into the aortic wall

Thrombosis had frequently, but not always occurred on the injured intima The amount of thrombus material varied widely (Fig 2) The thrombi were composed of platelets, with or without leucocytes, red blood cells, and fibrin (Figs 2 and 3) Some of the thrombi formed a layer of fairly even thickness, others were made up of columns of platelets with red and white blood cells in between (Figs 2 and 3) If fibrin was present, the amount was moderate It was found mainly at the base and the surface of the thrombi

In the pigs which were killed 2¼ 5 hours after the cannulation, the amount of fibrin associated with the thrombi was greater than in animals killed shortly afterwards Otherwise, the findings at the longer survival times were similar to those described

Electron microscopy of sections from uninjured areas of the thoracic aorta of both species showed intact endothelial cells A narrow space between the endothelial cells and the internal elastic lamina was filled with a basement membrane like amorphous mate

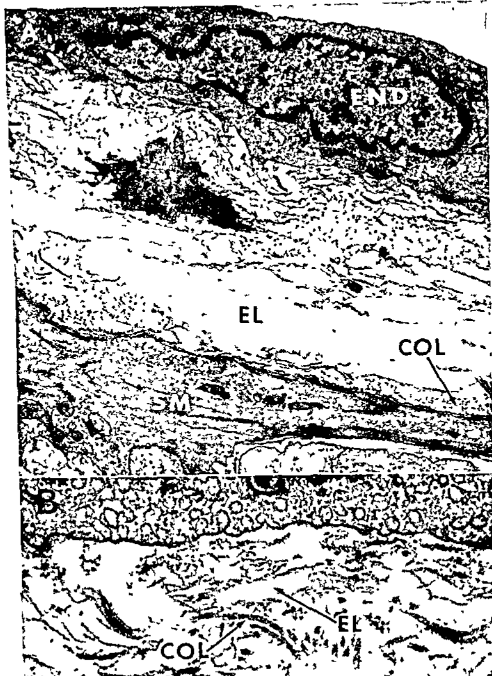


Fig 4 An uninjured part of the thoracic aorta of a rabbit A Endothelial cell (END), elastic lamina (EL), smooth muscle cells (SM), collagen fibres (COL), × 13,000 B Enlarged part of Fig 4A showing collagen fibres (COL) and elastic fibres (EL) beneath the endothelial cell, × 56,000

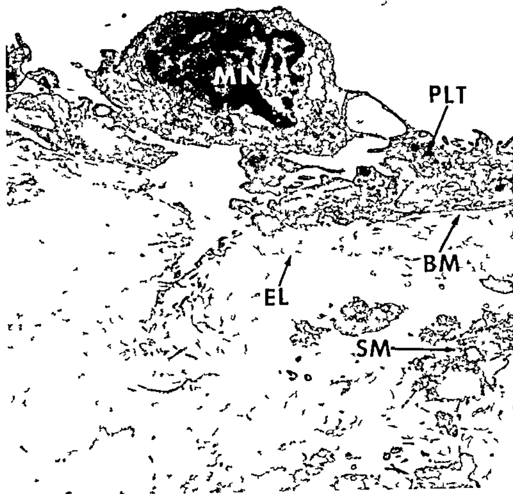


Fig 5 A blue streak in the ... The endothel al cells are m ss ng and replaced ... ell (MN) The platelets are in close contact ... f bres ⸌ ith as o-c ated m crofibr ls (EL) The structure at lower r ght is probably a damaged smooth muscle cell (SM) The components of the ⸌ascular wall are spread apart probably ind cat ng oedema × 8 300

r al elastic fibres with associated microfibrils collagen fibres and smooth muscle cells (Fig 4)

In sections from injured sites in animals killed 10 minutes or less after cannulation platelet and platelet leucocyte thrombi were found on the intimal surface which was usu ally devoid of endothelial cells (Figs 5 and 6) As in l ght microscopy the remaining parts of the innermost layers of the aortic wall showed increased space between their structures (Fig 5 In some of the sections there was a thin layer of platelets without fibrin (Fig 5) In other sections the layer of platelets was thicker and often fibrin was present at the base and on the surface of the thrombi (Fig 6)

The platelets at the base of the thromb were tightly packed and in close contact with basement membrane like material and elastic and collagen fibres (Figs 5 and 6) The platelets had pseudopods and these were frequently wedged in between the sub endo thelial structures (Fig 6) If in contact w th collagen fibres the platelets and particularly their pseudopods were swollen and their

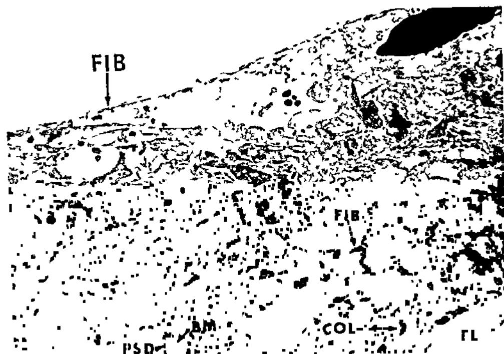


Fig 6 A blue streak in the aorta of a rabbit after Evans blue infusion and cannulation Here the platelets are not only in contact with basement membrane like material (BM) and elastic fibres (EL), but also collagen fibres (COL) The basal platelets have long pseudopods (PSD), some of which penetrate into the underlying tissue Fibrin (FIB) has formed among the basal platelets Near the luminal surface of thrombus the platelets are swollen and degranulated A fibrin layer (FIB) covers the thrombus, × 6,000

electron density reduced (Figs 6 and 7) Fibrin was usually not present on the damaged wall when the platelets were in contact with only basement membrane-like material or elastic fibres (Fig 5) When collagen fibres had been exposed, fibrin strands were in contact with both collagen fibres and swollen platelets and pseudopods (Figs 6 and 7)

In the middle of the thrombi, the platelets and their pseudopods had mostly retained their electron density and fibrin was only occasionally present (Fig 6)

Towards the periphery, the platelets were often loosely aggregated Some of the platelets were swollen and degranulated (Fig 6)

Polymorphonuclear leucocytes were frequently observed at the surface of the thrombi within crevices of the thrombi, or in between densely aggregated platelets (Fig 8). In contrast to the platelets, the leucocytes were not often in direct contact with the subendothelial structures of the aortic wall Most of the leucocytes were of the neutrophil type, a few were of other types The cytoplasm of some of the leucocytes was displaced towards one pole and the nucleus towards the other, as if the cells were in active motion Some of the leucocytes seemed to be engaged in phagocytosis of platelet material and several of the leucocyte granules had lost their electron-dense material (Fig 8)

DISCUSSION

The inner layers of the thoracic aorta in rabbits and pigs infused with Evans blue showed sharply demarcated blue streaks in the region reached by the carotid cannula From previous experiments with animals infused with plasma markers (11, 12, 16, 19), it is known that this pattern of staining does not occur "spontaneously" The blue streaks

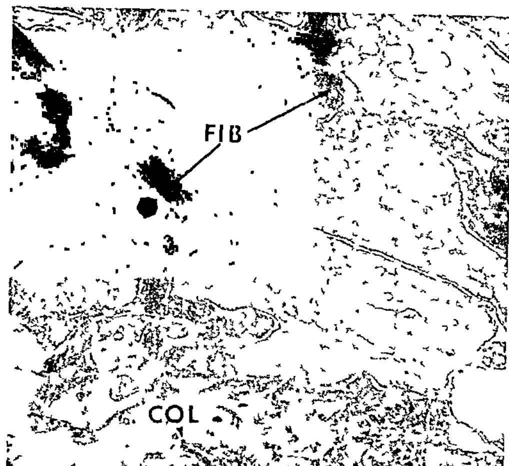


Fig 7 Enlarged port on of the base of the platelet thrombus sho n in F g 6 Fibr n (FIB) is present between the platelets and between the platelets and the injured vessel wall Collagen f bres (COL) are in close prox m ty to the fibr n and the platelets × 46 000

observed in the present study are therefore undoubtedly caused by the cannulation Since Evans blue is bound to plasma proteins (8) mainly albumin the blue staining in our ex periments indicates a localized accumulation of proteins in the inner layers of the aortic wall (6 13 16)

Microscopic examination of the blue areas also showed sharply demarcated signs of inti mal injury such signs were not observed in unstained areas This indicates that the increased accumulation of proteins was due to the injury caused by the cannula These ob servations support our previous conclusions that the accumulation of plasma proteins in areas of spontaneous inju to in creased permeab l ty caused ry (11) rather than to decreased clearance or increased leakage from the vasa vasorum (1 18) Further since the dye accumulated equally along the length of the streaky can nula lesions haemodynamic factors do not seem to influence the accumulation of the protein once the injury has occurred

In contrast to the slow development of the blue staining of spontaneous injury s tes in the aorta after infusion of Evans blue (11 16) the intense blue staining caused by the cannula developed very rapidly This d ffe rence is probably attributable to the greater severity of the cannulation injuries (cfr later)

The leakage of plasma proteins into the wall lasts at least 2 hours after the injury

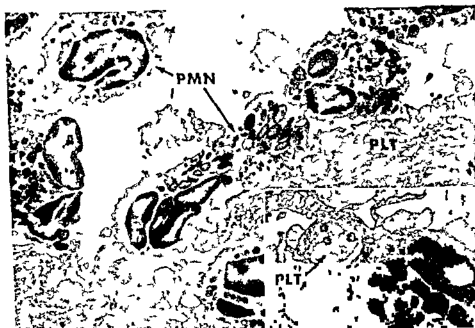


Fig 8 Surface of a platelet thrombus (PLT) with several polymorphonuclear leucocytes (PMN) Some of the leucocytes are elongated as if in active motion A few of their granules appear to have lost some of their electron dense material × 6 000 Inset A polymorphonuclear leucocyte which appears to have taken up platelet material (PLT) × 10 000

because the blue staining was similar whether the Evans blue was infused before or 45 minutes to 2 hours after the cannulation *Bjorkerud & Bondjers* (4) observed increased permeability to the Evans blue albumin complex at sites of defective endothelium caused by mechanical aortic injury 4 weeks previously

The intimal injury observed in the present study was characterized by loss of endothelial cells and commonly accumulation of platelets and other blood constituents Similar thrombus formation at sites of endothelial removal has been observed by other investigators (2 9 17 20 23) but studies of transfer of plasma proteins into the injured vessel wall have not been included In this study it appeared that the covering layer of platelets did not prevent the increased accumulation of plasma proteins in the vessel wall

At the injury sites there was increased space between the tissue components in the inner layers of the aortic wall indicating that the sites of albumin accumulation are in fact sites of oedema We have previously shown that at sites of spontaneous aortic injury there is a correlation between increased accumulation of plasma proteins and histological signs of oedema (16) In the case of spontaneous injury we have advanced the theory that the formed blood elements particularly the platelets are primarily responsible for the vascular injury and that the increased leakage of plasma into the aortic wall is of secondary nature (11 16) We did observe however an association between the number of leucocytes in the intima and the degree of oedema (11) which could indicate that the intimal leucocytes were causally related to the oedema In the present study oedema of the aortic wall was present at the injury sites even without leucocytic invasion of the intima suggesting that the previously observed association between intimal leucocytes and oedema is not necessarily a simple cause effect relationship

The fact that thrombi were not found in

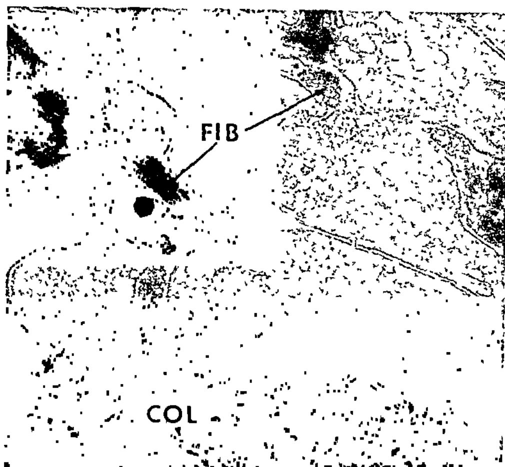


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In contrast to the slow development of the blue staining of "spontaneous" injury sites in the aorta after infusion of Evans blue (11, 16), the intense blue staining caused by the cannula developed very rapidly This difference is probably attributable to the greater severity of the cannulation injuries (cfr later).

The leakage of plasma proteins into the wall lasts at least 2 hours after the injury

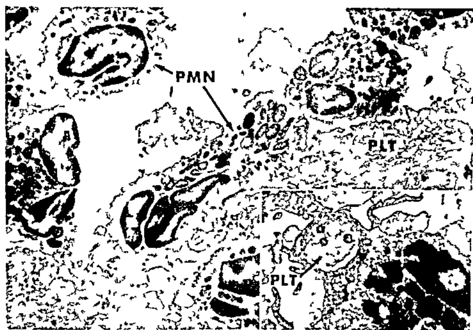


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some areas of denuded aortic wall may indicate that haemodynamic factors influence the occurrence of platelet thrombi on an exposed platelet activating surface However, the platelets could have been there prior to the time of examination, but had disappeared before the sections were taken *Baumgartner* (2) found that, if rabbit aortas were examined immediately after endothelial stripping platelet thrombi covered most of the surface, while examination of the injured vessel after 40 minutes showed very little platelet material

The platelets at the base of the thrombi were in close contact with basement membrane-like material, elastic fibres with associated microfibrils, and collagen fibres This is in accordance with our previous observations in "spontaneous' lesions (10, 11) Only platelets or platelet pseudopods in contact with the collagen fibres were swollen and had reduced cytoplasmic electron density Whether this difference is due to the activation of coagulation of collagen (see below) or to qualitative differences in the interaction between platelets and the various subendothelial structures (3) cannot be decided

Fibrin was present at the base of some of the thrombi as early as 5 minutes after cannulation This was found particularly at sites where collagen fibres had been exposed, an observation which may be explained by the fact that collagen is a strong activator of coagulation (15, 21, 22) In studies involving the lower portion of the aorta and the femoral arteries, fibrin was not observed at the base of the thrombi (3, 9, 20), a fact probably related to the scarcity of intimal collagen fibres in this region (20)

The leucocytes were localized on the surface and within crevices of the thrombi or among densely aggregated platelets They were, however seldom in contact with the structures of the aortic wall This indicates that the leucocytes accumulated after the platelets It has been shown that platelets contain a factor that is chemotactic for leucocytes (14)

These experiments indicate that brief contact of a cannula with the endothelium of the aorta is sufficient to cause injury of the intima in either rabbits or pigs The innermost vascular layer appears to be extremely vulnerable to this type of injury In general, the morphology of the injury sites is similar to but more extensive than, the advanced stages of the "spontaneous" lesions described in previous papers (10, 11, 16)

This work was supported in part by grants from the Medical Research Council of Canada, MT 1309, the Ontario Heart Foundation, and the Norwegian Research Council for Science and the Humanities

The skilful technical assistance of Mrs Marian Larsen is gratefully acknowledged

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Acta path. microbiol scand Sect A, 82 648–654, 1974

MYOCARDIAL DAMAGE IN CHILDREN AND ITS RELATION TO CORONARY ARTERY LESIONS

ERKKI PESONEN, M D

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Signs of fresh myocardial damage, as evidenced by the fuchsinorrhagic method, and thickenings of the inner vascular layers of the coronary arteries were found in a series of autopsies on children The myocardial changes were more prominent in the hearts of children with infections and congenital heart defects than in those with other diseases The extent of the *damage was significantly correlated with the thickness of the inner vascular layers* These observations support the view that the vascular wall can thicken rapidly The parallel occurrence of the myocardial damage and thickenings suggests a common aetiology

A frank heart infarction is rare in children (3, 17) On the other hand, multifocal myocardial necroses seem to be common in dying children (2) and probably play a role in the mechanism of death (33)

Even newborn infants may show extensive thickening of the inner vascular layers (intima plus musculoelastic layer) of the coronary arteries The size of these thickenings in young subjects and their morphology suggest that they may develop rapidly in response to some injurious agents (27) It has been shown before from the same basic autopsy material that thickenings appear in association with infectious diseases (27)

In this study the association between coronary artery changes ... myocardial lesions was investigated ... us between these findings and sought

MATER...

The series, ... children, was

Received 22

Group 1 comprises 31 infectious cases Of these 12 were septic In two cases there was a generalized viral infection Almost all the children (29 cases) had suffered from pneumonia terminally

Group 2 comprises 26 cases of congenital heart defects (CHD) Cases of patent ductus arteriosus only were not included in this group

Group 3, comprising 33 cases, forms the con*trol group* The members of this group were not suffering from infections at death, and did not have CHD Death was due to the neonatal respiratory distress syndrome, neonatal haemorrhagic diseases, congenital malformations other than CHD, or malignant diseases

Group 4, comprising 11 cases of CHD complicated by infection, was excluded from the comparison of the three main groups

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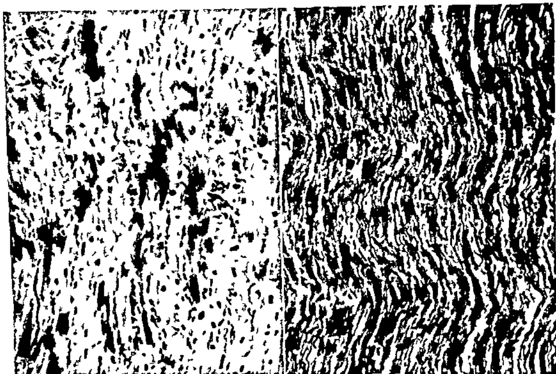


Fig 1 Classification of the myocardial changes as revealed by the fuchsinorrhagic technique × 480
a Grade one Occasional red stained cells appear black in the figure
b Grade three All the muscle cells are affected

coronary arteries were cross sectioned semiserially at 200 μm intervals

Sections 5 μm thick were stained with Verhoeff Van Gieson s stain to show fibrosis and elastic tissue Further, the papillary muscles were stained with the fuchsinorrhagic method of *Lie et al* (22) to show myocardial necroses in the early stages A reference section with histologically confirmed myocardial necrosis was included in every batch of slides stained Tissue calcifications were shown with the von Kossa method

The histological investigation was made without knowledge of the diagnostic group Fibrosis of the papillary muscles was estimated visually and scored from zero to three In grade one fibrosis occasional thin strands of collagen were observed

In grade two there were large focal areas of fibrosis In grade 3 over 50 per cent of the section was fibrotic

Fuchsinorrhagic changes in the myocardium were scored from zero to three where changes had occurred they were visible in several tens of cells In grade one small red bands were seen in the myocytes (Fig 1a) and occasional muscle cells were entirely red In grade two the bulk of the myocytes are coloured red In grade three the muscle cells are totally red (Fig 1b) The site of the change in the papillary muscle was recorded as subendocardial apical or central

From semi serial transverse sections of the right and left coronary arteries one section from each side showing the greatest area of the inner vascular layers was chosen for morphometric studies The areas of the media the part of the musculo elastic layer between the internal elastic lamina (IEL) and media and the layers on the luminal side of the IEL were measured planimetrically The length of the IEL was measured by the coordinatographic method (28) These measurements were transformed mathematically to give the dimensions of the arteries in the uncontracted state with (hypothetically) uniformly thick arterial layers and dimensions were used for comparing the arteries The methods has been described in detail previously (27, 28)

The data of the fuchsinorrhagic and fibrotic changes in the right and left papillary muscles and in the different diagnostic groups were examined by analysis of variance Stepwise regression analysis was used to study the correlation between fuchsinorrhagic changes and the thickness of the arterial wall layers Other factors used in this

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In this study the association between coronary artery changes and focal myocardial lesions was investigated Correlations between these findings and the autopsy diagnoses were sought

MATERIAL AND METHODS

The series, consisting of 101 unselected autopsied children, was divided into four groups

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Group 4, comprising 11 cases of CHD complicated by infection, was excluded from the comparison of the three main groups

The median for postnatal age was 9 days The oldest patient was 12 years and 4 months of age

The hearts were fixed in phosphate buffered 10 per cent formalin The anterior papillary muscles from the left and right ventricles and the first 1 to 3 cm of both main coronary arteries were embedded in paraffin For histological preparations the papillary muscle was sectioned longitudinally from the tip to the base, the part of the ventricular wall on which it was inserted was included in the section The reason for including the beginning of the chorda tendinae in the sections was to ensure that the central parts of the muscle were investigated and to avoid tangential sections Both

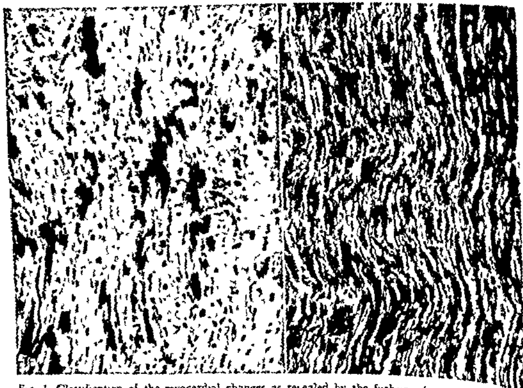


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TABLE 1 *The Percentages of Cases of Fibrosis in the Different Diagnostic Groups, when the Highes Score for Fibrosis in Either of the Papillary Muscles Is Taken into Account*

Fibrosis score	0	1	2	3
Infections	90 per cent (28)	3 per cent (1)	6 per cent (2)	0 per cent (0)
CHD	65 per cent (17)	15 per cent (4)	8 per cent (2)	12 per cent (3)
Controls	82 per cent (27)	9 per cent (3)	6 per cent (7)	3 per cent (1)
All	80 per cent (72)	9 per cent (8)	7 per cent (6)	4 per cent (4)

Numbers of cases in parentheses

study to explain the variance of the thickness of the arterial wall layers were the logarithm of the sum of the two radii, gestational age, postnatal age, the presence of infection, and the presence of congenital heart defect The variable to be explained in the regression model was the logarithm of the sum of the thicknesses of the corresponding layers on the right and left Details of the mathematical treatment have been published before (27)

RESULTS

Fibrosis was seen in 22 and fuchsinorrhagia in 72 of the 101 cases Calcifications were seen in only four cases two of them were associated with CHD, one case belonged to the group of infections, and one to the control group

Fibrosis

Fibrosis was more frequent in the right than in the left papillary muscle (p<0 02)

The cases with CHD differed from those with infections when the highest score for fibrosis in either of the papillary muscles was taken into account (p<0 05) The result was the same if the scores of the two papillary muscles were summed The scores for fibrosis in the different disease groups are shown in Table 1

Fuchsinorrhagia

Fuchsinorrhagia was more frequent in the right papillary muscle The p values for the differences were subendocardial<0 005,

TABLE 2 *The Percentages of Fuchsinorrhagic Scores in the Different Diagnostic Groups in the Endocardium of the Left Papillary Muscle*

Fuchsinorrhagic score	0	1	2	3
Infections	42 per cent (13)	23 per cent (7)	29 per cent (9)	6 per cent (2)
CHD	38 per cent (10)	23 per cent (6)	23 per cent (6)	15 per cent (4)
Controls	81 per cent (27)	9 per cent (3)	3 per cent (2)	3 per cent (1)
All	56 per cent (50)	18 per cent (16)	19 per cent (17)	8 per cent (7)

Numbers of cases in parentheses

apical <0.02, central (i e in the middle of the papillary muscle) not significant, and if the highest scores in the muscles were compared without regard to site <0.005

The frequency of the scores for subendocardial fuchsinorrhagia in the left anterior papillary muscles is shown in Table 2

In the subendocardial part of the left papillary muscle fuchsinorrhagic colouring was found by analysis of variance to separate the group with CHD very significantly ($p<0.001$) and the group with infections almost as significantly ($p<0.005$) from the control group The group with infections did not differ significantly from that with CHD

In the right papillary muscle subendocardial fuchsinorrhagia was most frequent in the CHD group A p value of less than 0.02 was obtained for the difference between CHD and infections

In the left papillary muscle as a whole the greatest change separates the groups with heart anomalies and infections ($p<0.05$) from the control group On the right side the greatest change separates the CHD group from both the group with infections and the control group ($p<0.05$)

Fuchsinorrhagia and the Thickeness of the Arterial Layers

The variance of the logarithm of the sum of the thickness of the intimal plus musculo-elastic layers in the right and left coronary arteries is correlated almost significantly ($p<0.05$) with the most severe fuchsinorrhagic change in the left papillary muscle in the regression model The other significant factors are logarithm of the sum of the outer radii ($p<0.001$) gestational age ($p<0.005$), and infections ($p<0.025$)

The fuchsinorrhagic muscle changes are not significantly correlated with the variance in the thickness of the media

DISCUSSION

Some hours after myocardial injury changes are evident in ordinary histological speci mens Several methods have been proposed for the detection of early muscular injury (4, 14 19, 26, 32) Those based on enzyme histo chemistry are probably the most reliable, but they cannot be used with paraffin sections (16)

The fuchsinorrhagic method of Lie et al 1971 (22) was adopted in this study The advantage of this method is its selective stain ing of early changes in the myocardium in or dinary paraffin sections

Circulatory insufficiency, where the blood cannot carry enough oxygen to meet the needs of myocardial metabolism, produces necrosis of the heart muscle cells (20, 31) In dying children small multifocal myocardial necroses seem to be very common and probab ly play a role in the mechanism of death (33) These necroses are probably due to hypoxia and hypoperfusion (2 8, 33) and, in hyper trophied hearts, to coronary insufficiency (18) Septic infection also seems to be one of the causal factors (7, 28)

The subendocardium is the most vulnerable part of the myocardium (9) The myocardial infarction is usually situated subendocardially in cases where there is no recent occlusion (23) The subendocardium receives its blood supply through penetrating arteries from the epicardial vessels (10) The papillary muscles were studied because they lie in the deepest endocardial region Infarction, especially of the papillary muscles and subendocardium is seen in a high proportion of children who die of congenital heart defects (8, 11, 13) In this study, old (fibrotic) and fresh (fuchsinorrhagic) signs of myocardial damage were also found in the papillary muscles in association with CHD In the group with infections signs of damage were abundant but were all fresh.

In this series of predominantly neonatal cases the preponderance of changes in the papillary muscle on the right side was not unexpected The high resistance of their pulmonary vessels is a feature peculiar to newborn children (15) Many infants had neonatal respiratory distress syndrome and about one third of the whole series (29 patients) had pneumonia In these conditions



Fig 2 Gross section of the left coronary artery of a boy who died of pneumonia at the age of four months The inner vascular layers, the musculo-elastic layer and the intima (bar in the figure) are thick and well developed × 800

the right ventricle has to perform unexpectedly heavy work against the vascular resistance and therefore sustains injury (23)

The arterial changes seem to be caused by the same factors which initiate myocardial damage Hypoxaemia leads to an increase in the mucopolysaccharides of the vessel wall (12) *In association with viral infections* there is swelling of the capillary wall (5) In the great arteries viral disease is associated with changes judged to be pre arteriosclerotic (6) As shown here and in a previous study (27) infections are associated with thickening of the inner vascular layers of the main coronary arteries

Infections and acute myocardial damage in the left papillary muscle have separate explanation powers in the stepwise regression analysis on the thickness of the inner vascular layers This result is interpreted as showing that infection exerts its effect by a mechanism different from that of other factors producing myocardial damage

The factor common to myocardial and coronary artery changes seems to be tissue swelling due to inhibition of tissue metabolism (21) When the ischaemic heart is perfused post mortem large areas do not fill with the perfusing fluid, especially in the inner aspects of the left ventricle The endothelial swelling of the capillaries (29) and the swelling of the muscle cells round the capillaries prevent the perfusate from reaching all parts of the myocardium (1) This reduces the oxygen supply to many small areas of the myocardium Histologically multifocal myocardial necroses are seen In the great arteries intimal thickening is due to tissue swelling and smooth muscle cell proliferation (27)

The parallel occurrence of acute myocardial damage and thickenings of the inner vascular layers does not necessarily indicate that they develop simultaneously or at the same rate The arterial thickening might be thought to precede and be partially responsible for the necroses in the myocardium The necroses seen in dying children might appear especially in association with narrowed arteries in a simultaneous hypoxic and hypotensive shock state However, theoretical calculations show that stenosis of the coronary artery does not affect coronary blood flow until it exceeds 30 per cent (24) Thus, in the majority of cases in the present series the arterial narrowing cannot have led to the necroses, although in some individual cases this may have been a contributory cause (Figure 2) But comparable swelling of the endothelial cells of the capillaries probably plays a role in producing the myocardial lesions (29 30)

The correlation between the fresh myocardial damages and the thickenings of the main coronary arteries suggests that a) at least some of the vascular thickenings develop rapidly and simultaneously with the myocardial changes and b) the two types of change have a common aetiology

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Fig 2 Gross sect on of the left coronary artery of a boy \ ho died of pneumonia at the age of four months The inner vascular layers the musculo elastic layer and the intima (bar in the f gure) are thick and well developed × 800

the right ventricle has to perform unexpectedly heavy work against the vascular resistance and therefore sustains injury (23)

The arterial changes seem to be caused by the same factors which initiate myocardial damage Hypoxaemia leads to an increase in the mucopolysaccharides of the vessel wall (12) In association with viral infections there is swelling of the capillary wall (5) In the great arteries viral disease is associated with changes judged to be pre arteriosclerotic (6 As shown here and in a previous study (27) infections are associated with thickening of the inner vascular layers of the main coronary arteries

Infections and acute myocardial damage in the left papillary muscle have separate explanation powers in the stepwise regression analysis on the thickness of the inner vascular layers This result is interpreted as showing that infection exerts its effect by a mechanism different from that of other factors producing myocardial damage

The factor common to myocardial and coronary artery changes seems to be tissue swelling due to inhibition of tissue metabolism (21) When the ischaemic heart is perfused post mortem large areas do not *fill with the perfusing fluid* especially in the inner aspects of the left ventricle The endothelial swelling of the capillaries (29) and the swelling of the muscle cells round the capillaries prevent the perfusate from reaching all parts of the myocardium (1) This reduces the oxygen supply to many small areas of the myocardium Histologically multifocal myocardial necroses are seen In the great arteries intimal thickening is due to tissue swelling and smooth muscle cell proliferation (27)

The parallel occurrence of acute myocardial damage and thickenings of the inner vascular layers does not necessarily indicate that they develop simultaneously or at the same rate The arterial thickening might be thought to precede and be partially responsible for the necroses in the myocardium The necroses seen in dying children might appear especially in association with narrowed arteries in a simultaneous hypoxic and hypotensive shock state However theoretical calculations show that stenosis of the coronary artery does not affect coronary blood flow until it exceeds 30 per cent (24) Thus in the majority of cases in the present series the arterial narrowing cannot have led to the necroses although in some individual cases this may have been a contributory cause (Figure 2) But comparable swelling of the endothelial cells of the capillaries probably plays a role in producing the myocardial lesions (29 30)

The correlation between the fresh myocardial damages and the thickenings of the main coronary arteries suggests that a) at least some of the vascular thickenings develop rapidly and simultaneously *with the* myocardial changes and b) the two types of change have a common aetiology

Acta path microbiol scand Sect A, 82 655–662, 1974

LOBULAR CARCINOMA IN SITU OF THE BREAST WITH DUCTAL INVOLVEMENT

Frequency and Possible Influence on Prognosis

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Out of 52 cases of lobular carcinoma in situ, 41 presented extralobular ductal involvement This seems to be of no prognostic importance Isolated occurrence of extralobular ductal involvement is not likely, and the demonstration of such lesions would require further examinations in order to detect lobular carcinoma in situ It is presumed that the cells in lobular carcinoma in situ and those seen in extralobular ductal involvement have a common genesis

In 1941 *Foote & Stewart* defined lobular carcinoma in situ as "cancer originating in lobules and terminal ducts" In 1968, *McDivitt et al* gave the following definition "Lobular carcinoma is used to designate a type of carcinoma thought to arise from the duct apparatus of the lobule, the intralobular duct and its terminal ramifications" This definition is now universally accepted

Involvement of extralobular ducts is not expressly included in this definition, although it is not incompatible herewith Only a few authors mention briefly, but clearly, extralobular ductal involvement in cases of lobular carcinoma in situ (*Antonius & Jones* 1963, *Bässler* 1969, *Hamperl* 1971, *Ozzello* 1971, *Hamperl* 1972, *Haagensen et al* 1972) *Fechner* (1972), on the other hand, gave a more detailed description of extralobular ductal involvement but his series comprised mainly invasive lobular carcinoma The object of the present study was to evaluate the frequency and, if possible, the influence on the prognosis of extralobular ductal involvement in lobular carcinoma in situ

MATERIAL AND METHODS

The material consisted of 52 cases of lobular carcinoma in situ of the breast and has been described earlier (*Andersen* 1974) The study included only cases of extralobular ductal involvement of the same monotonous cytological type and nature of growth as that characterizing the cellular population and the nature of growth in lobular carcinoma in situ All cases with hyperplasia of the ductal epithelium, as seen in fibroadenomatosis, and cases with myoepithelial hyperplasia, were excluded It has been established that the ducts involved were extralobular, whereas the size of the individual ducts was not assessed The majority of the specimens were stepwise sectioned and/or in certain areas serially sectioned, so that between 10 and 75 sections were obtained from each block The study was retrospective and consequently, the number of blocks available was stationary and the series could not be supplemented Hence, because of the number of tissue blocks available (1 6 per breast specimen), the results obtained must be considered as minimum figures

Received 2 x 74 Accepted 2 x 74

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RESULTS

Extralobular ductal involvement was demonstrated in 41 cases (78 per cent) The involvement was (1) parietal, (2) solid

Re (1) The parietal ductal epithelial proliferation appeared as a layer of epithelium, about 5 to 10 cells deep, most often ending luminally in the original columnar epithelial cells These were more or less flattened cells with a dark cytoplasm, contrasting sharply with the large polygonal proliferating cells which, on the other hand, had a pale and often vacuolized cytoplasm In the basal portions of the epithelium, the preserved myoepithelial cells were found, forming a discontinuous cellular layer inside the so called basal membrane (Figs 1, 7) Saccular epithelial proliferations protruded occasionally into the adjacent stroma, the proliferations almost resembling a string of beads (Figs 3 11) In a few specimens the ductal affection was restricted to the area around the outlet of the lobule and in some cases small accumulations of the large pale cells were situated in between the original epithelium (Fig 5) In some cases luminal papilliferous epithelial proliferations without supporting stroma were seen These epithelial proliferations consisted often of the original dark columnar epithelial cells, but some large pale proliferating cells could also appear in the papilliferous elements (Fig 4).

Re (2) The solid ductal epithelial proliferation was in some cases the only phenomenon found (Fig 2), in other cases it was associated with papilliferous epithelial proliferation Areas with a tendency to cribriform configuration were also seen No cases of direct transition from solid to parietal type were found, even though the two types of proliferation were often seen in the same specimen The solid epithelial proliferation seemed to be present only in small lactiferous ducts with direct transition into lobular carcinoma in situ, whereas parietal proliferation could also be seen in larger lactiferous ducts, even without clearly demonstrable transition into lobular carcinoma in situ Therefore, the lumen was often obliterated without any remnants of the original columnar epithelium in cases of solid epithelial proliferation On the other hand, the basal myoepithelium was preserved

The frequency of the various types of epithelial proliferation in extralobular ductal involvement is shown in Table 1

Hence, in 36 cases parietal epithelial proliferation was seen, whereas it was solid in 4 cases and in 1 both solid and papilliferous.

In only one case was extralobular ductal involvement found without demonstrable transition into lobular carcinoma in situ, whereas in 8 cases there were areas both with and without direct detectable transition into lobular carcinoma in situ Apart from one case with solid and one with papilliferous epithelial proliferation, the remaining 7 cases presented parietal epithelial proliferation When this affection was present, in particular in larger lactiferous ducts, deeper sectioning within a limited area could suddenly reveal outlets of lobules with epithelial proliferation, complying with the criteria for lobular carcinoma in situ (Fig 6)

In 7 cases the primary slides presented

Fig 1 Lactiferous duct with parietal epithelial proliferation of the lobular type The original columnar epithelial cells are seen luminally as a flattened layer of cells with dark cytoplasm In the basal part there are a few myoepithelial cells In between, large pale proliferating cells are seen, with corresponding large round nuclei, most often with distinct nucleoles In this portion of the epithelium no stratification is seen Haematoxylin eosin 456 ×

Fig 2 Lactiferous duct with solid epithelial proliferation of the lobular type Haematoxylin-eosin 456 ×

Fig 3 Lactiferous duct with parietal epithelial proliferation of the lobular type, but with saccular epithelial protuberances in the adjacent stroma Alcian blue (*Eskelund* 1957) 456 ×

Fig 4 Lactiferous duct with parietal epithelial proliferation of the lobular type Furthermore there are papilliferous epithelial proliferations, some consisting exclusively of the original dark columnar epithelial cells others also of the large pale proliferating cells Haematoxylin-eosin 456 ×




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Fig. 1. Lactiferous duct with parietal epithelial proliferation of the lobular type. The original columnar epithelial cells are seen luminally as a flattened layer of cells with dark cytoplasm. In the basal part there are a few myoepithelial cells. In between, large pale proliferating cells are seen, with corresponding large round nuclei, most often with distinct nucleoles. In this portion of the epithelium no stratification is seen. Haematoxylin-eosin. 456 ×.

Fig. 2. Lactiferous duct with solid epithelial proliferation of the lobular type. Haematoxylin-eosin. 456 ×.

Fig. 3. Lactiferous duct with parietal epithelial proliferation of the lobular type, but with saccular epithelial protuberances in the adjacent stroma. Alcian blue (*Eskelund* 1957). 456 ×.

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TABLE 1 *Types of Extralobular Ductal Involvement in 52 Cases of Lobular Carcinoma* in situ

Parietal	
Without saccular proliferations	19
With saccular proliferations	12
Both with and without saccular proliferations	5
With papilliferous proliferations	9
Solid (papilliferous and cribriform)	17

ductogenic epithelial proliferations of lobular type without diagnostic lobular lesions indicating lobular carcinoma in situ, but by deeper sectioning into the blocks such changes were revealed (Figs 8, 9, and 10)

In 11 of the 52 cases, extralobular ductal involvement could not be detected, in spite of exhaustive study of the remaining material However, it was possible in only 2 of the 11 cases to demonstrate that the terminal lactiferous duct was definitely not affected, and it was not possible to detect any direct continuity between the lobule and the terminal lactiferous duct in the remaining 9 cases

Amongst the 52 cases of lobular carcinoma in situ, 8 with ipsilateral invasive breast carcinoma were found during the follow up period (2–28 years) Seven of these were found amongst 41 cases with extralobular ductal involvement, whereas 1 was detected amongst 11 cases without extralobular ductal *involvement* Out of the 52 patients with lobular carcinoma in situ, 15 patients developed invasive carcinomas, 5 previous to and 10 during the follow-up period Twelve of these cases were found amongst 41 cases with extralobular ductal involvement and 3 amongst 11 cases without extralobular ductal involvement The difference is not statistically significant

DISCUSSION

The frequency of extralobular ductal involvement in the present series of lobular carcinoma in situ is in complete agreement with that found by *Fechner* (1972) in a series comprising mainly invasive lobular carcinoma

This agreement between the frequency in a series of lobular carcinoma in situ and a series mainly comprising invasive lobular carcinoma, and the results of the prognostic part of the present study, indicate that the extralobular ductal involvement exerts no prognostic influence on any subsequent development of invasive breast carcinoma

On the other hand, the occurrence of these very characteristic ductal lesions without concurrent lobular carcinoma in situ, must give rise to a more exhaustive investigation of the remaining tissue In the present study I succeeded, in 7 cases, in detecting lobular carcinoma in situ by deeper sectioning Hence, out of the 3299 breast specimens investigated for otherwise benign breast disease, I found no case of ductal proliferation of lobular type without it being possible to detect lobular carcinoma in situ in the blocks by sectioning of the specimens

Therefore, extralobular *ductal involvement* will in actual practice be seen only in cases of lobular carcinoma in situ

Ozzello (1971) pointed out that, in lobular carcinoma in situ the mammary ducts would

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Fig 5 Small lactiferous duct with localized epithelial proliferation of the lobular type and luminal flattening of the original dark columnar epithelial cells Haematoxylin eosin 456 ×

Fig 6 Lactiferous duct with parietal epithelial proliferation of the lobular type There is a small lobular outlet with lesions indicating lobular carcinoma in situ Haematoxylin eosin 182 ×

Fig 7 Lactiferous duct with abrupt transition between simple hyperplastic epithelium (right) with oblong nuclei cytoplasmic decapitations in the luminal part and a tendency to stratification and the precancerous epithelium (left), which in other respect corresponds to Fig 1 Haematoxylin eosin 456

Fig 8 Lactiferous duct with parietal epithelial proliferation of the lobular type The section originates from the primary slide and the lesion gave rise to deeper sectioning Haematoxylin eosin 182 ×

Figs 9 and 10 Same case as Fig 8 The finding after deeper sectioning was an area presenting lobular carcinoma in situ arising from the original lactiferous duct Haematoxylin eosin 182 × (Fig 9) and 456 × (Fig 10)



Fig 11 Terminal lactiferous duct with pronounced saccular epithelial proliferations of the lobular type Lobular carcinoma in situ will often be found in the proximity of such ducts Haematoxylin-eosin 456 ×

often present clusters of large pale cells 'comparable to the better differentiated cells of lobular carcinoma and show essentially the same cytoplasmic organization However, in spite of the fact that it was possible by serial sectioning to detect continuity between these cell proliferations in the ducts and lobular carcinoma in situ he was of the opinion that the exact nature of this lesion was unclear (neoplastic preneoplastic, or hyperplastic?)

Light microscopy studies, however, do not seem to contradict a common origin of the cells in lobular carcinoma in situ and the cells in the involved extralobular ducts, and the extralobular ductal involvement seems to occur secondarily to lobular carcinoma in situ

CONCLUSIONS

1 Extralobular ductal involvement was seen in 41 (78 per cent) out of 52 cases of lobular carcinoma in situ

2 No significant relationship was found between the detection of extralobular ductal involvement and the frequency of invasive breast carcinoma

3 Extralobular ductal involvement without simultaneous lobular carcinoma in situ is not likely, and isolated detection of the lesion would, therefore, require further examination

4 The frequently detected continuity between the proliferating cells seen in lobular carcinoma in situ and the proliferating cells occurring in extralobular ductal involvement indicates a probable common origin

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Fig 11 Terminal lactiferous duct with pronounced saccular epithelial proliferations of the lobular typ Lobular carcinoma in situ will often be found in the proximity of such ducts Haematoxylin-eosin 456 ×

often present clusters of large pale cells "comparable to the better differentiated cells of lobular carcinoma and show essentially the same cytoplasmic organization" However, in spite of the fact that it was possible by serial sectioning to detect continuity between these cell proliferations in the ducts and lobular carcinoma in situ, he was of the opinion that the exact nature of this lesion was unclear (neoplastic, preneoplastic, or hyperplastic?)

Light microscopy studies, however, do not seem to contradict a common origin of the cells in lobular carcinoma in situ and the cells in the involved extralobular ducts and the extralobular ductal involvement seems to occur secondarily to lobular carcinoma in situ

CONCLUSIONS

1 Extralobular ductal involvement was seen in 41 (78 per cent) out of 52 cases of lobular carcinoma in situ

2 No significant relationship was foun between the detection of extralobular duct involvement and the frequency of invasiv breast carcinoma

3 Extralobular ductal involvement withou simultaneous lobular carcinoma in situ is n likely, and isolated detection of the lesio would, therefore, require further examina tion

4 The frequently detected continuity b tween the proliferating cells seen in lobul carcinoma in situ and the proliferating ce occurring in extralobular ductal involveme indicates a probable common origin

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Acta path microbiol scand Sect A, 82 663-664, 1974

TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting Linkoping, March 10, 1973

B Winblad & M Duchek THE VALUE OF FLUORESCENCE MICROSCOPY IN THE DETECTION OF TUBERCLE BACILLI

An important and tedious task in the pathological laboratory is the demonstration of acid fast bacteria in tissue sections Hagemann in 1937 was the first to report the advantages of a fluorescent dye

We have compared the efficacy of microscopical methods (Z N and A R – Auramine Rhodamine fluorescent dye) and cultivation (L J Lowenstein Jensen) on 87 specimens from uro genital organs of guinea pigs infected experimentally with a human strain of tubercle bacilli ($H_{37}Rv$)

Ziehl Neelsen and L J were carried out in the usual manner A R stained sections were examined in a Zeiss Large Universal Fluorescence Microscope with exciter filter BG 12/3 and barrier filters 47 or 50 At higher magnifications, epi illumination would show the bacteria more clearly than trans-illumination.

Comparative efficacy of the three methods used for the detection of tubercle bacilli showed that all three methods were positive in a total of 33 specimens In 23 specimens Z N staining was negative but using the A R method and culture, bacilli were demonstrated Among a further 25 cases in which Z N staining gave negative results, bacilli were demonstrable by the A R method exclusively in 14 cases and by culture exclusively in 11 cases Six specimens which were positive to staining methods were negative on culture No specimen was negative in fluorescence microscopy but positive to Z N staining

We conclude

Fluorescence staining is superior to Z N staining and comparable to cultivation.

The investigation confirmed that the A R method as compared with Z N staining involves a cleaner easier and more simple staining technique, it requires only dry objectives reduces the examination time and gives a higher percentage of positive results especially if tubercle bacilli are scanty

Received 6 vii 74

I Dahl & L Angervall CUTANEOUS AND SUBCUTANEOUS LEIOMYOSARCOMA AND ITS RELATION TO RETRO PERITONEAL LEIMYOSARCOMA

A series comprising 39 cases of superficial leiomyosarcoma was presented In 14 patients the tumour was located within the corium, in the additional 25 cases it was mainly located in the subcutis Thirty two patients (24 men and 8 women with a median age of 67 years) had a solitary tumour Seven patients had multiple tumours which were located subcutaneously Four of these patients had earlier been subjected to operation for a retroperitoneal leiomyosarcoma In the fifth patient, an inoperable retroperitoneal tumour was demonstrated by X ray but not verified histologically as a leiomyosarcoma Most cases ran a prolonged clinical course and the rate of local recurrence was high (about 30 per cent) Sixteen patients died of their tumour disease after intervals ranging from 1 month to 11 years Metastases were manifested in three of these who died after more than ten years

In a national series of superficial and deep soft tissue sarcomas hitherto collected during the period 1958 1963, sixty three (10 per cent) were leiomyosarcomas, thirty-one of these were located retroperitoneally In 5 of the retroperitoneal leiomyosarcomas, multiple tumours of soft tissue occurred Therefore we consider that patients with mult ple leiomyosarcoma of soft tissue should be examined for a retroperitoneal tumour

L G Kindblom, L Angervall & B Stener INTRAMUSCULAR MYXOMA

The authors presented 18 cases of intramuscular myxoma 16 of which had been collected during the last 12 years at the Department of Pathology, Sahlgren Hospital, Goteborg, Sweden The thigh was a site of predilection and, in most cases, their diameters measured 5 cm or less Histochemical studies using the Scott technique for acid mucopolysacharides, i e staining with Alcian with the addition of increasing concentrat indicated the presence of hyal was also found by

sections with bovine hyaluronidase and staining with Alcian blue and Toluidine blue at different pH s Angiographic, micro angiographic and histological studies showed that intramuscular myxoma is sparsely vascularized By way of comparison, the micro angiographic appearance of a richly vascularized myxoid liposarcoma was demonstrated, i e an important differential diagnosis of intramuscular myxoma Recurrence or metastasis were not demonstrated in any case of intramuscular myxoma Muscle resection or myoectomy is considered to be the adequate treatment

Acta path microbiol scand Sect A, 82 665–674, 1974

DISTRIBUTION OF LYSOZYME IN THE SERUM, URINE AND KIDNEYS OF AKR MICE DURING THE PATHOGENESIS OF LYMPHOCYTIC LEUKEMIA

A Biochemical, Histochemical and Lightmicroscopic Study

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The Institute of Cancer Research and the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, N Y 10032, USA

The distribution of lysozyme in the serum, urine and kidney homogenates was studied in normal young (less than 4 months) AKR mice, in old (more than 8 months) AKR mice that bore a spontaneous lymphocytic leukemia and in young AKR mice in whom this leukemia had been transplanted Much higher concentrations of lysozyme were detected in the kidney homogenates of the old mice than among the young mice However, after the injection of leukemic cells into the young mice, increased concentrations of lysozyme were detected in their kidneys as well Concentrations of lysozyme in the serum did not vary markedly in either age group or during the course of leukemic cell proliferation During the pathogenesis of the transplanted leukemia, morphological changes were observed within the proximal tubular cells of the kidneys by light microscopy, and by the cytochemical demonstration of lysozyme using an immunoperoxidase technique The number and size of dense and lysozyme positive granules of the proximal tubular cells increased concomitantly with the increase in lysozyme levels in kidney homogenates Lysozymuria developed in the advanced stages of the disease and was associated with morphological signs of tubular cell damage The findings of elevated lysozyme levels in leukemic mice are discussed as a phenomenon related to the stimulation of the reticuloendothelial system induced either by the injected leukemic cells or by the viral agent associated with this lymphocytic leukemia

One diagnostic tool for differentiating between various types of human leukemias is the determination of the concentration of lysozyme in serum and urine An elevated level of lysozyme in either or both of these body fluids is generally indicative of a neoplastic proliferation of lysozyme-containing cell types Hence a malignant proliferation of monocytic or myelocytic cells is accompanied by an increase in the concentration of lysozyme in the serum and urine, whereas neoplasms of lymphocytes i e, cell types devoid of lysozyme, are not generally associated either with elevated serum lysozyme levels or with lysozymuria *(18, 19, 28)*

An animal model for an acute myelogenous leukemia has been described in the case of the rat Shay chloroleukemia (24) As the lysozyme containing leukemic cells in this experimental leukemia proliferate, the concentration of lysozyme in the serum, urine and kid-

Received 19 ii 74 Accepted 19 iv 74

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sections with bovine hyaluronidase and staining with Alcian blue and Toluidine blue at different pH s Angiographic, micro angiographic and histo logical studies showed that intramuscular myxoma is sparsely vascularized By way of comparison, the micro angiographic appearance of a richly vascularized myxoid liposarcoma was demonstrated i e an important differential diagnosis of intramuscular myxoma Recurrence or metastasis were not demonstrated in any case of intramuscular myxoma Muscle resection or myoectomy is considered to be the adequate treatment

TABLE 1 *Concentration of Lysozyme in the Serum, Urine and Kidney Homogenates of Young and Old AKR Mice*

	Young normal AKR mice (<4 months)		Old leukemic AKR mice (>8 months)		Significance p value
	Mean ± SD	Number of animals	Mean ± SD	Number of animals	
Serum (µg/ml)	3.4±0.3	12	3.6±0.4	9	not significant
Urine (µg/ml)	<1.0	34	6.6±2.8	28	–
Kidney homogenate (µg/g wet weight)	75.0±8.0	6	212.0±13.0	6	<0.001

of which bore a spontaneous lymphocytic leukemia.

Concentrations of Lysozyme in AKR Mice Bearing the Transplanted Leukemia

From 8 to 11 days after intraperitoneal inoculation of leukemic cells into young mice, there appeared the first signs of generalized leukemia, viz., *enlarged spleen and enlarged* inguinal lymph nodes. Fig. 1 shows the levels of lysozyme in the serum, urine and kidneys of these mice as determined during the course of the disease. Concentrations of lysozyme in the serum remained without any statistically significant deviation between 3.6–4.1 µg/ml throughout the course of leukemic cell proliferation.

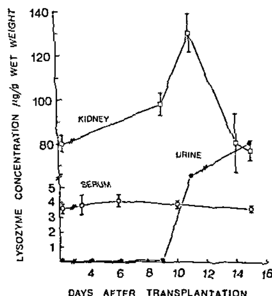


Fig. 1. Variations in the concentrations of lysozyme in the serum of 5, in the urine of 8, and in the kidney homogenates of 3 young AKR mice after transplantation of leukemia. Mean ± SD.

The level of lysozyme in the kidney homogenates, however, showed a notable variation. From day 0 to day 11 after transplantation of the leukemic cells the concentration of lysozyme in the kidney homogenates rose from 80 µg/g to 128 µg/g wet weight of kidney ($p<0.01$). Between days 11 and 15 this concentration dropped to about 80 µg/g. Because there had been a sharp increase in the weight of the kidney due to extensive infiltration of non-lysozyme containing leukemic lymphocytes, the actual elevation in the levels of lysozyme per gram of kidney tissue was probably even greater than the figures would indicate. Not until 11 days after transplantation of the leukemic cells was lysozyme detected in the urine. The maximum level of lysozymuria was reached on day 14, with a concentration of about 80 µg/ml. Moreover, during the course of the disease a decrease was observed in the concentration of lysozyme in the spleen.

The Relationship between Lysozymuria and the Length of Survival Time of AKR Mice with the Transplanted Leukemia

The most marked variations in the concentrations of lysozyme among the mice with the transplanted leukemia were observed in their urine. Furthermore, the lysozymuria phase bore a relationship both to the passage nu-

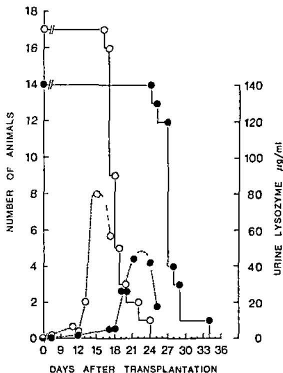


Fig 2 Graphic representation of the relationship between length of survival time (solid lines) and concentrations of lysozyme in the urine (dotted lines) of young AKR mice after transplantation of leukemic lymphocytes Note especially the close relationship between lysozymuria and length of survival time after the first passage (●——● ● --●) and after the third passage (O——O O – O) of leukemic cells Each point represents the mean concentration of lysozyme in the urine of at least 8 mice

ber of the leukemic cells and to the length of survival time of the mice after transplantation (Fig 2) In the first passage of tumor cells, the injection of 1×10^6 leukemic cells into a group of 14 young AKR mice, resulted in a median survival time of 27 days (range 24–34 days) Tumor cells in the third passage had an apparently more "malignant" effect, the median survival time being 18 days (range 16–24 days) No change in the length of survival time was noted after the third passage of tumor cells

Tracing the concentration of urinary lysozyme after these cell passages revealed different excretion patterns In the first passage of tumor cells, no signs of lysozymuria could be detected in the earlier stages of the disease and peak values of 50–80 ug/ml were not reached until after 21 days In the third passage of tumor cells, however, peak levels were reached already after 15 days In both instances, maximum lysozymuric concentrations preceeded the death of the mice by 3 6 days

Changes in the Morphology of the Kidney in AKR Mice Bearing the Transplanted Leukemia

The changes in the concentration of lysozyme both in the kidney homogenates and in the urine that occured after the transplantation of leukemia, were correlated with changes in the morphology of the kidney, primarily with changes in the structure of the proximal tubules (Figs 3–6). When studied by light microscopy the proximal tubular cells of the kidney of young normal AKR mice (Fig 3) revealed the characteristic cytoplasmic constituents, i e , few dense granules, as well as a number of small vacuoles of comparable size in the intermediate zone and just under the brush border, respectively In the basal part of the cell could be seen elongated regular rodlike structures whose shape and distribution were consistent with the configuration of mitochondria

By the 9th to the 11th day after injection of the leukemic cells i e , at the time when the maximum lysozyme concentration had been reached in the kidney homogenates both the number and the size of the dense granules in the proximal tubular cells were increased (Fig 4) The diameter of these granules now measured about 1 μ and they appeared in numbers of up to 10 15 granules per cell At this same time, moreover, the first leukemic infiltrates were visible in the kidneys

By day 14, i e , on the day of maximum lysozymuric concentration, the morphology of the proximal tubules had undergone marked changes (Fig 5) Leukemic infiltrates had become more abundant especially around the small blood vessels The proximal tubules, moreover, appeared to be in various stages



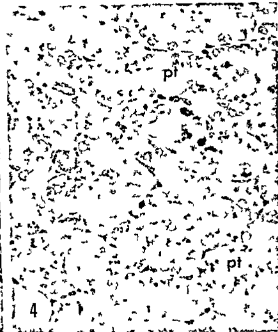


Fig 3 Kidney of a normal young AKR mouse In the proximal tubule cells (pt) a few small dense granules and vacuoles can be observed just beneath the brush border 430 ×

Fig 4 Kidney of an AKR mouse 11 days after transplantation of leukemia Note the increased number of dense granules present in the cells of the proximal tubule (pt) 430 ×

of degeneration The dense granules that had been so prominent on days 9 and 11 had now disappeared and vacuoles of varying size were seen This vacuolization became further prominent 15 18 days after tumortransplantation at this time large vacuoles occupied most of the proximal tubular cell cytoplasm (Fig 6) Also large areas of tubular necrosis had become visible

The electron microscopic appearance of the proximal tubular cells 11 days after tumor transplantation is shown in Fig 7 The number and size of membrane limited dense cytoplasmic bodies with dense matrix (lysosomes) were increased in the area between the brush border and the nucleus The matrix of these dense bodies was homogeneous or coarse granular and in some a minute vacuolization was present

Cytochemical Localization of Lysozyme in the Kidneys of AKR Mice after Transplantation of the Lymphocytic Leukemia

Lysozyme was clearly demonstrated in the proximal convoluted tubules of the normal young AKR mice (Fig 8) In the sections stained by the immunoperoxidase technique and examined by light microscopy lysozyme appeared to be distributed diffusely throughout the cytoplasm as well as in some dense granules that lay in the apical region of the tubular cell The intensity of the diffuse staining of lysozyme in the tubular cells was greater 9 and 11 days after transplantation of tumor cells (Fig 9) Most striking however was the increase in the number of lysozyme positive granules after such transplantation The size and localization of these granules corresponded fairly well to the distribution and size of the granules that stained with methylene blue in the 1μ sections On days

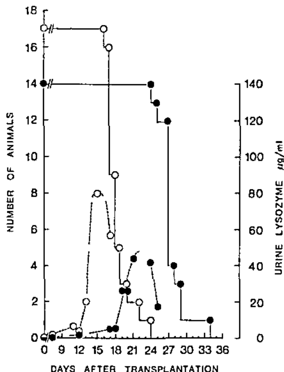


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Fig 7 Electron micrograph of a proximal tubule cell 11 days after transplantation of leukemia One large apical vacuole (av) containing some dense material and several enlarged membrane limited cyto plasmic bodies (cb) with electron-opaque matrix (lysosomes) can be seen in the apical region of the cell Nucleus (n), brushborder (bb), and mitochondria (m) 26000 ×

a common denominator for these situations is a stimulation of the reticuloendothelial system (RES) Although stimulation of the RES by the tumor cells themselves is one possibility, *Perri* (20) suggested that other factors accompanying the growth of the tumor are actually responsible for the RES activation In fact, *Old et al* (16) have shown that some transplanted tumors stimulate the RES of their host through the action either of the tumor-associated bacteria or the virus like agents associated with the tumor cells As far as the present results are concerned, the presence of a viral agent, the so called Gross virus, in the leukemic lymphocytes is well documented (7) Similarly *Perri et al* (20) after injecting splenic cells of mice infected with Friend virus leukemia into Swiss mice observed "progressively increasing concentrations of lysozyme in kidney tissue, reaching a maximum 14 days after injection In spleens, however, a considerable decrease occured" Moreover, it has been demonstrated that the number and the functional activity of granulocytes and macrophages increases during the host's reaction to neoplasia and that granular enzymes are released from granulocytes and macrophages during the phagocytic process (4, 17) In previous studies it has been possible to trace exogeneously administered or endogenously produced lysozyme to cytoplasmic structures of the proximal tubular cells (10, 21) In the present study an increase in the number of tubular cell granules was observed simultaneously with an increase in the concentration of lysozyme in the kidney homogenates By electron microscopy the granules corresponded to enlarged lysosomes (14, 15) A similar size and distribution pattern was observed in the immunohistochemically visualized lysozyme-positive granules of the proximal tub-

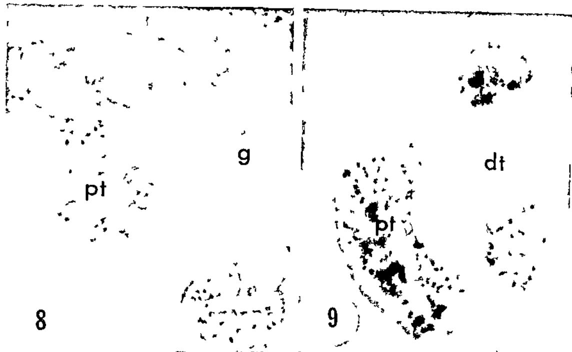


Fig 8 Cytochemical localization of lysozyme in the proximal tubule cells of a normal AKR mouse Lysozyme, stained here by an immunoperoxidase technique is present diffusely throughout the cytoplasm as well as within the cytoplasmic granules 1120 ×,

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ular cells These morphological results are in good agreement with earlier studies on the lysosomal localization of lysozyme within the kidneys as studied by differential centrifugation (25)

The fact that the serum levels of lysozyme did not increase significantly during the proliferation of the leukemia may be explained by the great capacity of the proximal tubular cells to reabsorb rapidly large quantities of lysozyme from the circulation (8) However in addition to the suggested increased [illegible]thesis of lysozyme by cells of the RES a[illegible]sed catabolism of lysozyme by the kidne[illegible]annot be ruled out Cell products releas[illegible] the proliferating leukemia cells ma[illegible] the capacity of protein disposal b[illegible] kidneys leading to accumulation of prot[illegible] (lysozyme) within the tubular cytoplasm [illegible]ing advanced stages of the leukemia the lysozyme positive enlarged granules of the proximal tubular cell cytoplasm disappeared In their place vacuoles of varying size occupying most of the cytoplasm became prominent These vacuoles were lysozyme negative when stained and studied by the immunoperoxidase technique The disappearance of lysozyme staining of the proximal tubular cytoplasm as well as the decrease in the concentration of lysozyme of kidney homogenates seemed to be a consequence of tubular cell damage with subsequent loss of tubular cell lysozyme in the urine The findings are in agreement with the studies on experimental lysozymuria induced by tubulotoxic chemicals such as mercuric chloride and sodium dichromate (1) Development of lysozymuria indicated final stages of the disease, as evidenced by a consistent relationship between lysozymuria and

survival time Death occurred about one week after the onset of lysozymuria

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[illegible] Dr *Elliott F Osserman* for his [illegible]

zyme antiserum, and to Mrs *Harriett Ansari* for her invaluable contributions during the microscopic phases of this study

Studies supported by Grant CA—02332 of the *National Cancer Institute* to Dr *Elliott F Osserman*

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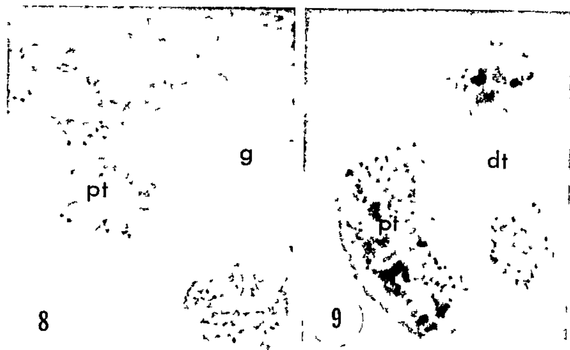


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ular cells These morphological results are in good agreement with earlier studies on the lysosomal localization of lysozyme within the kidneys as studied by differential centrifugation (25)

The fact that the serum levels of lysozyme did not increase significantly during the proliferation of the leukemia may be explained by the great capacity of the proximal tubular cells to reabsorb rapidly large quantities of lysozyme from the circulation (8) However in addition to the suggested increased net synthesis of lysozyme by cells of the RES, a decreased catabolism of lysozyme by the kidneys cannot be ruled out Cell products released from the proliferating leukemia cells may overtax the capacity of protein disposal by the kidneys leading to accumulation of proteins (lysozyme) within the tubular cytoplasm During advanced stages of the leukemia, the lysozyme-positive enlarged granules of the proximal tubular cell cytoplasm disappeared In their place vacuoles of varying size occupying most of the cytoplasm became prominent These vacuoles were lysozyme negative when stained and studied by the immunoperoxidase technique The disappearance of lysozyme staining of the proximal tubular cytoplasm, as well as the decrease in the concentration of lysozyme of kidney homogenates seemed to be a consequence of tubular cell damage with subsequent loss of tubular cell lysozyme in the urine The findings are in agreement with the studies on experimental lysozymuria induced by tubulotoxic chemicals such as mercuric chloride and sodium dichromate (1) Development of lysozymuria indicated final stages of the disease, as evidenced by a consistent relationship between lysozymuria and

surv val time Death occurred about one week after the onset of lysozymuria.

I am grateful to Dr *Elliott F Osserman* for his cont nued support throughout this work. I wish also to extend my thanks to Dr *P J Prosser* for his generos ty in supplying the rabb t anti mouse lysozyme antiserum and to Mrs *Harriet Amarel* for her invaluable contribut ons during the many phases of th s study

Stud es supported by Grant CA—02332 of the *Nat onal Cancer Institute to Dr Elliott F Osserman*

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Acta path microbiol scand Sect A, 82 675–682, 1974

CONCENTRATION AND IMMUNOHISTOCHEMICAL LOCALIZATION OF LYSOZYME IN GERM-FREE AND CONVENTIONALLY REARED RATS

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The biochemical activity of lysozyme and the immunohistochemical cellular localization of lysozyme were studied in germ free and conventionally reared rats In comparison to conventionally reared rats, germ free rats had significantly lower concentrations of lyso zyme in the salivary gland, duodenum, colon spleen, bone marrow and serum Lysozyme concentrations were also lower, though not significantly so in the lung and the liver By contrast, increased levels of lysozyme were observed in the thymus and ileum of germ free in comparison with conventionally reared rats No lysozyme was detected in the urine or in the intestinal content of the ileum or the colon of either group Lysozyme was detected by immunoperoxidase staining in the proximal tubules of the kidney, in the alveolar macrophages and in the Paneth cells of the small intestine in both germ free and con ventionally reared rats However, in the Paneth cells of the ileum an increased intracellular concentration of lysozyme was observed in the germ free rats Moreover in the thymus of germ free rats, lysozyme positive reticular cells were observed that could not be localized in the thymus of conventionally reared rats In addition to the observed low levels of immunoglobulins and complement, a relative lack of lysozyme could play a part in the increased susceptibility to infection of germ free rats

Since the discovery of lysozyme (muramidase, N acetyl muramide glycanohydrolase EC 3 2 1 17) by *Fleming* in 1922, the biologic function of lysozyme has been associated mainly with its antibacterial properties (7) It is generally agreed that lysozyme participates in the non specific defence mechanisms of the host against environmental microorganisms Increased concentrations of lysozyme in serum and some organs have been observed in animals after exposure to either live bacteria or to bacterial toxins (4, 6, 24) Furthermore the occurrence of lysozyme neonatally would indicate that the exogeneous environment has a role in the regulation of both concentration and distribution of lysozyme (18) It seems apparent that most of the circulating lyso zyme is derived from the turnover of leuco cytes of myelogenous origin (16)

Of special value as a source of comparative data in the study of host microorganism interactions is the germ-free (GF) organism In GF rats the concentration of lysozyme in serum is lower than that in conventionally reared (CV) rats, and the differences in this concentration are closely related to the dif-

Received 30 v 74 Accepted 26 vi 74

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ferences in blood leukocyte counts (15) After exposure to a conventional environment the number of circulating granulocytes and the concentration of lysozyme in the serum of GF rats return to normal

However, equally important in local antibacterial defence, as the level of circulating lysozyme is the distribution and concentration of lysozyme at the cellular level The present study reports comparative data on the concentration and immunohistochemical localization of lysozyme in GF and CV rats

MATERIALS AND METHODS

Germ free Sprague Dawley female rats were purchased from ARS/Sprague Dawley, Laboratory Park, Chagrin Falls Ohio, USA The rats were four weeks old, weighed from 75 to 90 g and were fed on an autoclaved regular Purina Chow 4 per cent diet Not until immediately before they were killed, the rats were removed from their germ free transporting isolator drum The GF state was verified by the absence of growth of aerobic and anaerobic bacteria of cecal contents The concentration of gamma globulins in the serum was determined by serum electrophoresis on cellulose acetate strips in barbital buffer, pH 8 6 (Microzone®, Beckman Instr Co) Conventionally reared Sprague Dawley rats matched with the GF rats for sex, age and weight, were kept in conventional cages with eight animals in each cage and were also fed a Purina Chow 4 per cent commercial diet

Bone marrow from the femur, pieces of liver, spleen, kidney, duodenum, ileum and colon, the intestinal content of the ileum and colon, as well as the serum and the urine were tested for the concentration of lysozyme When these determinations involved organs the organs were rapidly removed under aseptic conditions, weighed and stored at 20° C until assayed The tissues were homogenized in 1 5 (w/v) 0 07 M phosphate buffer pH 6 3 with ten strokes of a Potter Elvehjem homogenizer with a teflon pestle The homogenate was first frozen and thawed five times, and then centrifuged at 3000 rpm for 30 minutes Concentrations of lysozyme were determined from the supernatant by the lysoplate method using purified rat lysozyme as standard (22) The immunoperoxidase technique for demonstration of lysozyme has been described else here (17) Briefly, using this technique the tissues were fixed in 10 per cent phosphate buffered neutral formalin and embedded in paraffin as for routine histologic study Sections cut to 6 μ thickness were deparaffinized in xylene, rehydrated in serial ethanols, and washed in 0 01 M phosphate buffered *saline (PBS)*, pH 7 2 for 10 minutes The immunoperoxidase (immunoglobulin enzyme bridge) method for localizing lysozyme was performed as follows, the tissue sections were covered for 30 minutes each with 1) rabbit anti rat lysozyme antiserum, 2) with sheep anti rabbit gamma globulin antiserum and 3) with rabbit anti peroxidase antiserum Finally the sections were incubated with horse radish peroxidase (Sigma Co Type VI) at a concentration of 250 μg/ml The lysozyme was visualized by staining of the peroxidase according to the method of *Graham* & *Karnovsky* (3), using 3,3 diaminobenzidine and H_2O_2 as substrate To test the specificity of the lysozyme staining the primary specific rabbit anti rat lysozyme antiserum was replaced with PBS, with rabbit anti hen egg white lysozyme antiserum, or with rabbit anti rat lysozyme antiserum absorbed with purified rat lysozyme Only when this control staining was completely negative compared to a positive staining with the anti rat lysozyme antiserum, was the result interpreted as a significant positive Occasionally the peroxidase stained sections were counterstained with Harris haematoxylin (Fisher Sci Co)

RESULTS

The germ free condition of the GF rats was verified by the lack of bacterial growth in their caecal contents as well as by a markedly reduced fraction of gamma globulin (albumin/gammaglobulin, mean±SD 16 5±3 2) in comparison with the CV rats (albumin/gamma globulin 4 8±0 7), as determined by *electrophoresis*

The range in the concentration of lysozyme detected in the serum, the urine and in the homogenates of various organs in *both GF* and CV rats is presented in Table 1

No lysozyme was found in the urine or in the intestinal contents of either the GF or CV rats The concentration of lysozyme in the lung and liver homogenates were about the same in both groups The concentration of lysozyme in the bone marrow however, was lower in the GF than in the CV rats, at a level of significance of $p<0\,02$ The levels of lysozyme in the serum salivary gland, spleen, kidney, duodenum and colon were significantly lower $p<0\,01$, in the GF rats than in the CV rats On the other hand

TABLE 1 *Concentration of Lysozyme (μg/g Wet Weight) in Various Organs of Germ Free (GF) and Conventionally Reared (CV) Rats*

	GF rats (n=6)	CV rats (n=8)	GF/CV × 100	Significance p value
Serum (μg/ml)	1 5± 0 05	3 1± 0 3	48	< 001
Salivary gland	1 3± 0 1	2 0± 0 2	65	< 001
Thymus	7 0± 1 2	4 1± 2 6	171	< 05
Lung	144 0+21 4	170 0±41 9	85	not sign
Liver	2 4± 0 4	2 6+ 0 7	92	not sign
Spleen	28 7± 5 0	59 0±21 5	49	< 01
Kidney	76 2±10 0	175 0±73 0	43	< 01
Bone marrow of femur (μg/g femur)	38 0+10 1	48 6±15 2	78	< 02
Duodenum	11 9± 1 4	30 0±12 0	40	< 005
Ileum	92 0±17 7	39 0±14 2	236	< 001
Colon	2 6± 0 3	4 3± 1 2	60	< 01
Intestinal content ileum	0	0	–	–
colon	0	0	–	–
Urine	<1	<1	–	–

Mean±SD (number of animals)

the concentration of lysozyme in the thymus and ileum homogenates was markedly higher in the GF rats ($p<0\ 05$ and $p<0\ 001$ respectively)

Immunohistochemical Localization of Lysozyme

By the immunoperoxidase technique, lysozyme showed the same pattern of distribution in the kidney, lung and small intestine of both CV and GF rats (3 rats studied in each group) However, lysozyme positive cells that could not be located in the thymus of CV rats were detected in the thymus of the GF rats In neither group was lysozyme detectable in the salivary gland, in the spleen or in the liver

Kidney

The localization of lysozyme in the kidney was the same among the GF rats as among the CV rats Fig 1 Specific staining appeared only in the proximal tubular cells and whereas staining there was predominantly diffuse throughout the cytoplasm, many lysozyme positive cytoplasmic granules were seen as well The quantitative estimation of lysozyme as judged from the intensity of the staining in simultaneously stained sections of CV and GF rat kidneys, revealed a definitely less intense staining in renal tubular cells of the GF rats

Lung

In the examination of pulmonary tissue, each microscopic field, whether from GF or CV rats, contained about the same number of alveolar macrophages In both types of rats, the alveolar macrophages stained intensively for lysozyme and no clear difference in the intensity of the staining between the two groups of rats could be discerned (Fig 2) In both groups the alveolar macrophages appeared both as fixed and as free floating cells in the alveolar space No other part of the pulmonary tree exhibited any specific staining for lysozyme

Intestine

Lysozyme was detected in the Paneth cells of the duodenum the jejunum and the ileum of both GF and CV rats Whereas in both groups of rats the number of Paneth

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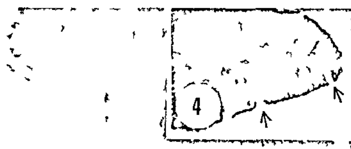
No lysozyme was found in the urine or in the intestinal contents of either the GF or CV rats The concentration of lysozyme in the lung and liver homogenates were about the same in both groups The concentration of lysozyme in the bone marrow, however, was lower in the GF than in the CV rats, at a level of significance of $p<0 02$ The levels of lysozyme in the serum, salivary gland, spleen, kidney, duodenum and colon were significantly lower $p<0 01$, in the GF rats than in the CV rats On the other hand

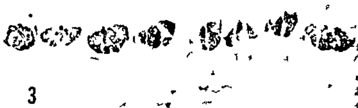
Fig 3 Ileum of a GF rat Lysozyme is predominantly seen in the Paneth cells at the base of the crypts of Lieberkuhn Magnification 350 ×

Fig 4 Ileum of a GF rat that shows a thin line of faint staining along the luminal surface of the epithelial cells Note the negatively stained globlet cells (arrows) Magnification 560 ×

Fig 5 Thymus of a GF rat stained by haematoxylin and eosin At the corticomedullary border large reticular cells with convoluted nuclei can be seen Magnification 560 ×

Fig 6 The same type of clustered thymic cells as seen in Fig 5 stained for lysozyme by the immunoperoxidase technique These cells show a definite staining for lysozyme in their cytoplasm







half to two times the diameter of the surrounding lymphocytes

DISCUSSION

Germ free animals completely devoid of a living microbial flora have been shown to be unusually susceptible to a variety of infectious agents including certain viruses and bacteria (27 30) Although the extent of susceptibility varies among different species of GF animals in GF rats the phenomenon has been explained as the result of an inadequate development of the lymphatic system (10) and lowered concentrations of immunoglobulin (33) complement (25) and properdin (11)

As originally reported by *Fleming* (7) lysozyme had a lytic effect on many bacteria mostly known as apathogens It has since then become apparent that lysozyme participates in bacteriolytic reactions also against a variety of pathogens Thus together with

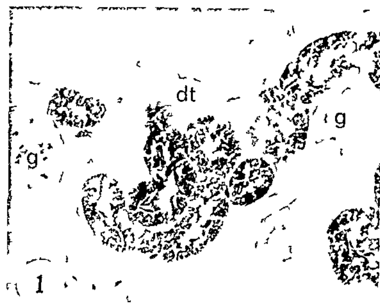


Fig 1 Kidney of a GF rat stained *for lysozyme by the* immunoperoxidase technique Staining is localized to proximal tubular cells Glomeruli (g) and distal tubular cells (dt) show no staining Magnification 350 ×

Fig 2 Lung of a GF rat Positive staining is seen in alveolar macrophages Magnification 350 ×



cells per each crypt of Lieberkuhn was about the same those in the GF rats stained with far greater intensity In both groups the staining *was restricted primarly to granules found* throughout the cytoplasm of the Paneth cells (Fig 3) Besides a weak possibly non specific lysozyme staining along the luminal surface of the epithelial cells of the intestine in both groups of rats (Fig 4) no other part of the intestinal wall showed any detectable staining for lysozyme

Thymus

No part of the thymus of CV rats stained *for lysozyme However in GF rats* clusters of 8 10 lysozyme positive reticular cells were found in the cortex close to the medullary zone of the thymus The nucleus of these lysozyme positive cells was either convoluted or bandshaped and the lysozyme staining was diffuse throughout the cytoplasm (Figs 5 6) The size of these cells was about one and one

tween anionic mucous material and cationic lysozyme, possibly of Paneth cell origin, on the surface of the epithelium

The low concentration of lysozyme of thymus homogenates, as well as the absence of lysozyme in thymic cells of CV rats agrees well with earlier findings on the thymus of mice (28) and rats (17) However, the increased concentration of lysozyme in the thymic homogenates and the number of lysozyme positive cells observed in the thymus of the GF rats is interesting By morphologic criteria these cells were reticular cells and fit the description of some of the reticular cell types characterized by *Sainte Marie* & *Leblond* (26) They described thymus reticular cells that had an extremely elongated and convoluted nucleus and which, in the present study, were lysozyme-positive in the GF rats but lysozyme negative in the CV rats Electron microscopic studies have separated the reticular cells of the thymus into epithelial and mesenchymal reticular cells (20) The mesenchymal cells have acid phosphatase positive lysosomes Because most lysozyme, in general, is a lysosomal enzyme, an association between lysozyme and these phagocytic mesenchymal reticular cells in the thymus of GF rats is also likely However, why lysozyme disappears from such cells once conventional rearing is introduced remains to be explained

Although this study provides information on the concentration and localization of lysozyme in GF rats, conclusions about the significance of lysozyme in the resistance to environmental microorganisms must be made with caution Other cellular events such as the decreased capacity to mobilize leukocytes (1), or cellular conditions such as the decreased concentration of immunoglobulins and complement cannot at present be distinguished from the possible role of lysozyme in determining the eventual course of infection in GF animals

I gratefully acknowledge the support of Dr *Elliott F Osserman* throughout the present study I am grateful to Dr *Harry Seneca* for bacteriological verification of the germ free state of the animals

Studies supported by grant CA 02332 of the *National Cancer Institute* to Dr *Elliott F Osserman*, Columbia University, New York, N Y USA

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THE PERMEABILITY PATHWAYS IN THE WALLS OF INTESTINAL SUBMUCOSAL ARTERIOLES IN ACUTE ANGIOTENSIN-INDUCED HYPERTENSION IN RATS

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the University Institute for Experimental Medicine, Copenhagen, Denmark

In rats with acute angiotensin induced hypertension, segments from intestinal submucosal arterioles dilated and permeable to plasma components were studied in the electron microscope The permeability pathways were found to be endothelial gaps measuring from 900 Å to 29 microns, discontinuities of the endothelial basement membrane, fenestrae in the internal elastic lamina and discontinuities of the luminal basement membrane of the media Most of the endothelial gaps were shown to be dilated endothelial junctions, and colloidal carbon and formed elements of the blood were found passing through the gaps In media, carbon was found extra and intracellularly if the smooth muscle cells were severely damaged Penetration of carbon into the adventitia was not found when the adventitial basement membrane of the media was intact The changes are probably caused by mechanical factors secondary to the development of dilatations

Mesenteric arterioles react with constrictions and dilatations when chronic or acute hypertension is induced in rats (*Byrom* 1954) Under the light microscope, the dilatations seem permeable to plasma components, while this does not apply to the constrictions (*Giese* 1964) Electron microscopic studies have showed that the increased permeablity is partly caused by development of gaps in the endothelial barrier (*Olsen* 1971, *Goldby* & *Beilin* 1972)

Endothelial gaps could arise either from destruction of the endothelial cells or from dilatation of endothelial junctions The aim of the present work is by use of the electron microscope to give further elucidation of the morphological basis for the increased permeability

MATERIALS AND METHODS

The experimental and tissue fixation techniques were exactly as described in a previous paper (1974a), except that the rats were injected intravenously with angiotensin II instead of physiological saline

Animals Nine white Wistar male rats weighing 190 260 grams were used

Angiotensin Hypertensin Ciba 500 micrograms in 100 ml physiological saline.

Angiotensin was injected intravenously at a dose

Received 24 iii 74 Accepted 10 vi 74

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of 0 5 1 0 microgram every fifth minute for three hours Following an injection of angiotensin the blood pressure rose from the normal pressure of 90–100 mm Hg to 150–170 mm Hg The blood pressure was registrated continously With use of an abdominal window and a stereo microscope, as described previously, it was possible to follow the development of dilatations and constrictions on *the submucosal arterioles from the upper half of* the jejunum and to ensure that both were fixed unchanged Fifteen minutes before fixation and death the rats received an intravenous injection of colloidal carbon After fixation, performed by perfusion technique in seven rats and by in vivo immersion technique in two rats, twenty dilated segments with deposits of carbon were dissected out under the stereo microscope The dilatations were after final preparation sectioned serially and examined in the Hitachi HS 7S electron microscope While the original diameter (before angiotensin injections) of these arteriolar segments was 60–100 microns, the dilated segments measured 90–150 microns in diameter

RESULTS

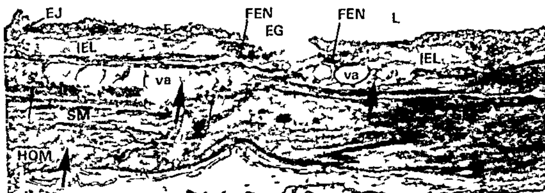
In the dilated arteriolar segments, carbon focally penetrates through all layers of the vascular wall *In all cases of penetration, this occurred through discontinuous parts of the* endothelial layer, basement membranes, internal elastic lamina and media Carbon was not found in caveolae or vesicles of the endothelial cells, neither was it found intracellularly Only in the smooth muscle cells of media was carbon sometimes found intracellularly and then in severely injured cells

The endothelium The endothelial gaps, which are the discontinuous parts of the *endothelial layer, measured from 900 Å to* 29 microns (Figs 1, 2, 5, 6, 7) Gaps were found in all except four dilatations The number of gaps in the lining endothelium varied from 1 to 20 per dilatation They were limited by endothelial cells with intact cell membranes In many cases it was possible in the serial sections to follow the opening up or dilatation of a normal endothelial junction to a real gap in the endothelial lining (Figs 1, 2, 3, 4) In a few cases the same change could be seen to develop *from already dilated junc*tions measuring 170 300 Å in width In these cases, it could be concluded that the discontinuity of the endothelium was a dilated endothelial junction In larger gaps, small islands of endothelium measuring 500 Å to 2 microns could be found (Fig 7) In all gaps, carbon particles and/or cellular blood components were observed (Figs 1, 5, 6, 7) Corresponding to the endothelial gaps, penetration of carbon particles, plasma and sometimes cellular blood components was seen in many cases (Fig 6) At these places, the internal elastic lamina was fenestrated and both the endothelial and the luminal basement membrane of the smooth muscle cells of media were discontinuous Beneath the endothelial cells limiting the endothelial gaps, the subendothelial space was dilated and in many cases carbon particles were observed penetrating the endothelium into the dilated subendothelial space, running luminally to the internal elastic lamina to a fenestra in the lamina and penetrating through the fenestra into the tunica media

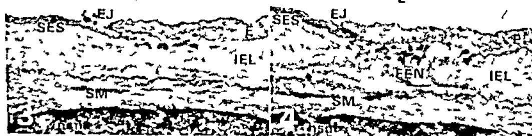
The internal elastic lamina Fenestrae in the elastic lamina (Figs 1, 5, 6) were greater

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Figs 1–4 The same part of an arteriolar wall from a dilated segment is seen in all four pictures Sections are cut serially and between Fig 1 and Fig 2 there is a distance of approximately 2 2 microns between Fig 2 and 3 approximately 5000 Å and between Fig 3 and 4 approximately 2000 Å The interendothelial gap (EG) seen in Fig 1 measures 1 5 microns in width Followed in serial sections it is seen gradually to close (Figs 2 4) to form a normal endothelial junction (EJ) in Fig 4 The fenestrae in the internal elastic lamina (FEN), *which are seen in Fig 1, allow carbon and plasma* to penetrate into the media The fenestrae have disappeared in Fig 2 and 3, but another one is seen in Fig 4 The subendothelial space (SES) is dilated in all four sections In Fig 1 there is distortion of media with dilated intercellular space (thick arrows), vacuoles (va), fine granulated homogenous areas (HOM) and dispersed carbon particles (arrows) A normal junction (EJ) is seen at left In Fig 2 carbon is found between the smooth muscle cell (SM) and the adventitial basement membrane of media (ABM) E endothelial cell, IEL internal elastic lamina, L lumen, nsm nucleus of smooth muscle cell Perfusion fixation Cacodylate buffered glutaraldehyde Uranyl *block* stained Fig 1 × 10,000 Fig 2 × 23 000 Fig 3 and 4 × 27,800



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than in normal arterioles varying from 4000 Å to 7 microns but the number of fenestrae seemed to be equal to that of normal arterioles Through the fenestrae, carbon, plasma and formed blood elements penetrated from the subendothelial space into the media if an endothelial gap was situated in the neighbourhood Such penetration seemed only to take place if discontinuity also existed in the endothelial basement membrane and in the luminal basement membrane of the smooth muscle cells of media

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Fig 5 A gap between two endothelial cells (E) is completely occupied by a mononuclear cell (MON) Carbon particles (arrow) are seen in the cytoplasm of this cell, which has a large nucleus (nu) The mononuclear cell is also partly penetrating through a fenestra in the internal elastic lamina (IEL) but there is no penetration of carbon or cellular blood elements into the media (M), and the basement membrane (BM) under the fenestra in the internal elastic lamina appears intact L lumen Immersion fixation Phosphate buffered glutaraldehyde × 29,500

Fig 6 The endothelial edge of an endothelial gap is seen (arrow) Part of a neutrophilic granulocyte (PLEU) is penetrating through the gap, through a fenestra in the internal elastic lamina (IEL) and a discontinuity of the basement membrane (BM), into the media (M) Carbon accompanies the leucocyte into the media (short arrow) E endothelial cell, L lumen, LEU leucocyte, RBC erythrocyte Immersion fixation Phosphate buffered glutaraldehyde × 22 000

Fig 7 Part of a very big endothelial gap is seen The edges of the gap are not seen in the picture Partly covering the internal elastic lamina (IEL), several small endothelial fragments (EF) are seen In the lumen (L), many blood platelets (THR) and parts of a neutrophilic granulocyte (PLEU) are seen Two small fenestrae (thick arrows) of the internal elastic lamina are present The media is severely damaged with remnants of more or less damaged smooth muscle cells (SM) The intercellular space (IS) is widened with fine granular homogeneous material Carbon (arrows) spreads mainly extracellularly Adventitial basement membrane (ABM) is intact and carbon does not penetrate to the adventitia (A) BM basement membrane va vacuole Perfusion fixation Cacodylate buffered glutaraldehyde Uranyl block stained 9,000

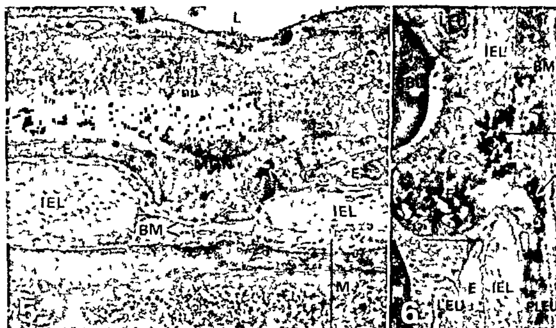
The tunica media Carbon particles and plasma were deposited in the intercellular spaces In cases with marked degenerations of the muscular cells, particles were also found intracellularly (Figs 1, 2, 7) The adventitial basement membrane was usually intact and carbon particles did not penetrate into the adventitia (Figs 2, 7)

DISCUSSION

In angiotensin induced hypertension, the increased permeability of intestinal submucosal arterioles is, according to the present findings, which are in agreement with those of *Suzuki et al* (1971), due to the formation of gaps in the endothelium If this condition is fulfilled, permeation takes place into the subendothelial space If penetration is to extend into the tunica media, discontinuities must have developed in the endothelial basement membrane, the internal elastic lamina and the luminal basement membrane of the smooth muscle cells Only if the adventitial part of the basement membrane surrounding the smooth muscle cells in the tunica media is discontinuous, will the penetration take place into the adventitia also In this way, no observations indicated a primary pinocytic transport as mentioned by *Ooneda et al* (1965)

In many studies of experimental hypertension, evidence for increased permeability without development of gaps is found In these cases, increased diffusion is thought to occur on transport through apparently morphologically normal junctions or as pinocytotic transport (*Moore et al* 1963, *Still* 1967 and 1968, *Giacomelli et al* 1970, *Huttner et al* 1970, *Kojimahara* & *Ooneda* 1970) Our observations do not exclude these permeability changes but it is evident that they could be responsible for only a very minor part of the increased permeability (1974c)

The vascular wall response to angiotensin, administered locally, is a vasoconstriction (*Tacek* 1967, *Crystal et al* 1970, *Douglas* 1970 The endothelial response is a contraction with opening up of interendothelial


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The development of gaps followed by great permeability changes, is based upon mechanical factors, secondary to the development of dilatations as assumed by *Giese* (1966) These changes are not directly due to the effect of administered angiotensin, but to the hypertension produced by it

Goldby & *Beilin* (1972) concluded that the endothelial gaps result from disruption of the cell body rather than from opening of intercellular junctions The results in the present study including the serial section technique showed that many of the gaps in the endothelium were dilated endothelial junctions In some cases however, it was not possible to decide whether gaps could also develop as a result of endothelial damage

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PORCINE SALMONELLOSIS: A COUNTERPART TO THE GENERALIZED SHWARTZMAN REACTION

Origin of Hyaline Material Precipitated in Minute Vessels

KNUT NORDSTOGA

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The hyaline or 'fibrinoid' material deposited in glomerular capillaries and minor vessels of the corium in association with swine salmonellosis, an experimental model of the generalized Shwartzman reaction, was found to be derived predominantly from disintegrated red blood cells Severe stasis with excessive vasodilatation preceded the erythrocytic damage Massive haemolysis did not seem to take place, the stagnant and tightly packed red cells in the engorged vessels were submitted to various types of disintegrating processes the ultimate result being partial lysis It is suggested that this erythrocytic debris corresponds to the hyaline masses which frequently stain as fibrin in conventional histological sections

Septicaemic salmonellosis in swine is frequently accompanied by fibrinoid necrosis in renal interlobular arteries and afferent glomerular arterioles together with widespread thrombosis in the same vessels and in glomerular capillaries These vascular lesions are compatible to the vascular alterations accompanying the generalized Shwartzman reaction (28, 29 39) and indicate that this phenomenon (GSR) plays an important role in the pathogenesis of porcine salmonellosis In the microcirculatory system where the lesions are, as a rule, most extensive in glomerular capillaries and small vessels in the skin the thrombo-occlusive masses are acellular and hyaline, often staining as fibrin (39) The aim of the present investigation was to study, in greater detail, the nature and origin of the hyaline material deposited in glomerular capillaries and minute skin vessels during septicaemic porcine salmonellosis

Received 10 ii 74   Accepted 5 v 74

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MATERIAL AND METHODS

Twenty four hour broth cultures of the strain of *Salmonella cholerae suis* used in previous experiments (39) were centrifuged and washed in saline, and saline bacterial suspensions inoculated into 8 experimental pigs (*Sus scrofa*), each weighing approximately 20 kgs (about 8 weeks old) In order to provide material with differing degrees of involvement the animals were injected with varying amounts of bacteria and killed at various times after the challenge by intravenous administration of 10 per cent mebumal

Pieces of kidneys and skin were fixed in a 10 per cent formaldehyde solution for at least 72 hrs, embedded in paraffin and sectioned at about 5 μ These stains were employed haematoxylin and eosin, the [illegible] Lepehne's haemoglobin stai[illegible] photungsti[illegible] drum's ac[illegible] scarlet blue (MBS) method (30) and the Ros indole reaction (15)



Fig 1 Early lesions in glomerular capillaries Erythrocytes some of which are stained red as fibrin, and a partly fibrillar fibrin stained material occlude the distended capillaries MSB × 1200

Material from superficial renal cortex and ear skin was preserved for examination in the electron microscope Fixation and infiltration processes were carried out by somewhat variable procedures Sections with slight or moderate tissue damage were preferred for the electron microscopic study The occlusive material in glomerular capillaries and minute skin vessels had apparently the same composition in all animals, but kidney tissue from one pig turned out to have lesions quite suitable for this study and also seemed to have been optimally fixed and infiltrated, hence, the illustrations are based on this material This animal received 1 ml of a saline bacterial suspension containing approximately 3500 × 10⁶ bacteria/ml (determined by the plate count method), the pig was killed 54 hrs after challenge The tissue was fixed in 3 per cent glutaraldehyde in Millonigs phosphate buffer for 1 hr at 4° C and post fixed in 2 per cent buffered osmic acid overnight After acetone dehydration and infiltration in Araldite and acetone ana partes for 4 hrs and in Araldite overnight at room temperature, the material was embedded in Araldite Electron microscopy was performed in a Siemens Elmiscope I A

RESULTS

All animals successively went into shock and developed severe dyspnoea and extensive cyanosis

Macroscopic Changes

External lesions consisted of pronounced cyanosis, particularly on the ears and the tail where incipient superficial necrotic skin alterations were sometimes present The cortical renal tissue was haemorrhagic and the kidneys were moderately enlarged in most animals

Light Microscopic Lesions

Kidney The glomerular capillaries were, as a rule, considerably dilated and stuffed with erythrocytes, the individual cells initially being distinctly visible and staining as normal erythrocytes However, the cells gradually lost their normal staining properties and were transformed into a hyaline material which frequently had the tinctorial characteristics of fibrin although the substance sometimes stained brownish rather than dark blue in PTAH sections At high magnification no components other than apparently morphologically unchanged erythrocytes, with distinct outlines, exhibited the staining properties of normal red cells In sections stained with Lepehne's method for haemoglobin the hyaline masses had frequently a more pale brownish tint than morphologically intact red cells Iron containing granules were not demonstrated in Turnbull stained sections

In advanced stages, the red cells seemed to fuse, leaving a homogenous or slightly fibrillar, material with all the staining properties of fibrin (Fig 1)

The endothelium seemed unaltered in the early phase of involvement The glomerular structures were gradually destroyed, and in advanced stages, the capsular spaces were dilated and contained blood and a proteinaceous fluid In the periglomerular interstitial tissue, varying numbers of extravasated red cells occurred

There was extensive hyaline droplet degeneration in all sections the changes were frequently most pronounced in the proximal tubules, the droplets exhibiting the staining characteristics of fibrin with PTAH as well as with the acid picro-Mallory and the [illegible] methods



Fig 2 Dilated glomerular capillaries containing tightly packed red cells and cellular debris probably originating from platelets (left hand side of the figure) × 8000

Fig 3 A glomerular capillary with red cells in early disintegration the cells have irregular outlines Incipient lysis is seen at the top and a platelet can be seen in intimate contact with the injured endothelium (arrow) × 14000



Fig 4 Distended glomerular capillary loops containing red cells and red cell debris, an erythrocyte of irregular shape in incipient disintegration is visible (arrow). × 6000

Fig 5 Extensive budding (arrows) and fragmentation of red cells in a glomerular capillary Note the uniform electron density of the spherules and the remaining parts of the erythrocytes, indicating that the fragmentation has occurred without evident loss of haemoglobin × 10000

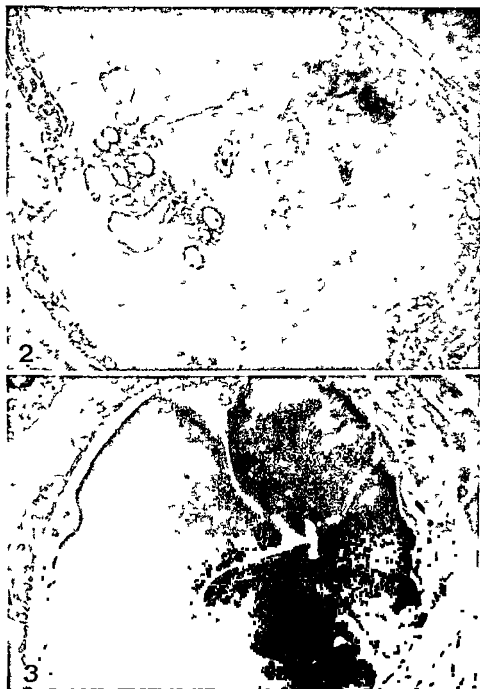


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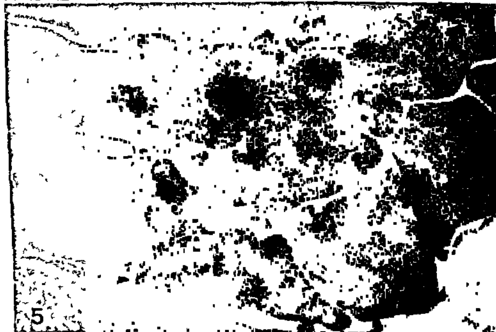


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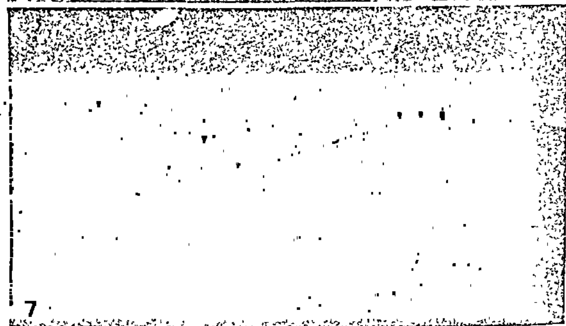


Fig 6. Red cells and red cell debris in a distended glomerular capillary × 27000.

Fig 7 Higher magnification of the framed part of Fig 7. A disintegrating erythrocyte with partial disappearance of the membrane (arrows) The material adjacent to the red cell has in some areas an ultrastructure which is very similar to the erythrocyte × 60000



Fig 8 Erythrocytes with somewhat varying electron density occlude a glomerular capillary together with a mass with relatively uniform electron density A partially lacerated red cells can be seen at the bottom to the left × 10000

Fig 9 Glomerular capillary loops packed with red cells which partly seem to have coalesced, the remaining part of the occlusive material consists of red cell debris Two platelets are indicated by arrows, cellular debris of uncertain origin (double arrow) × 6000

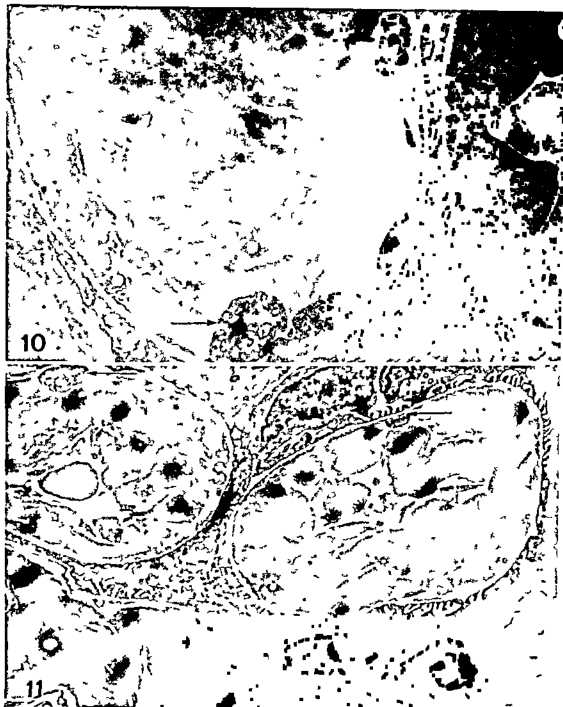


Fig 10 Distended glomerular capillaries containing thread like material in which a considerable lysis seems to have taken place partly fused erythrocytes to the right Two platelets adhere to the necrotic endothelium (arrows) × 8000

Fig 11 Distended glomerular capillary loops packed with remnants of red cells which have partly collapsed and formed a thread like material Two platelets stick to the necrotic capillary wall (arrows) × 4600



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tial fusion of red cells × 12000

Fig 13 Late change in a glomerular capillary which contains irregular occlusive masses of electron density material of high density is preferentially located at the periphery of the 14000

Skin Lesions in small skin vessels were similar to those occurring in glomerular capillaries.

Electron Microscopic Lesions

Kidney In the earliest stages of involvement the glomerular changes consisted exclusively of stagnation of erythrocytes in considerably dilated capillaries, sometimes platelets were trapped within the masses of intimately packed but morphologically nearly unchanged red cells (Fig 2) The breaking down of the red cells seemed to occur in various ways The first observable event in the red cells was circumscribed decrease in electron density, probably due to partial haemolysis (Fig 3) Incipient erythrocytic damage included also budding and fragmentation*, and laceration, sometimes giving rise to parallel structures simulating a thread like material Massive haemolysis did not seem to occur in this phase although the erythrocytic membranes were frequently obscured early in the transformation process (Figs 4–8) Fusion of damaged red cells seemed to take place occasionally

As the injury progressed, the disintegrating red cells appeared as irregular bodies of various shapes and sizes, and with varying electron density, sometimes similar to that of morphologically intact erythrocytes Later on, remnants of disintegrated cells formed confluent and occlusive masses in which delicate gaps, representing outlines of destroyed erythrocytes at times were discernible, larger spaces contained as a rule a flocculent or slightly granular material

Thrombocytes were often entangled in the occlusive material, they were nearly always found as individual structures, and never in aggregates, although they occasionally adhered to the necrotic endothelium or to the exposed basement membrane Leucocytes were only sporadically recognized In later developmental stages, lytic processes seemed to take place and, in such areas, the occlusive material frequently had distinctly differing electron density, also within circumscribed thrombus like structures (Figs 9–13)

Endothelial nuclei showed variable changes, including decreased and more even electron density than unaltered nuclei, the nuclei were sometimes partly loosened and compressed or displaced and squeezed together In advanced stages, the endothelial layer was totally necrotic and the basement membranes were subsequently exposed Severe lesions also included varying injury of the basement membranes and the epithelial cells, especially the foot processes which were often detached and disrupted, their remnants frequently being found in the distended capsular spaces together with a serum-like material

Skin The electron microscopic lesions in minute vessels in corium were principally the same as in glomeruli

DISCUSSION

The present investigation demonstrates that the major part of the hyaline masses which occlude glomerular capillaries and small skin vessels in porcine septicaemic salmonellosis, a model of the GSR (39), originates from stagnant and injured red blood cells clumped intimately together The erythrocytes undergo a series of disintegrating processes, the destruction seems to occur in various ways which exhibit characteristics similar to certain types of profound *in vivo* and *in vitro* erythrocytic damage (3), including the reported alterations in circulating red cells in association with human renal cortical necrosis and polyarteritis nodosa (6) Whether fusion of red cells, which has been demonstrated in *in vitro* experiments (1), also occurred prior to the disintegration, could not be determined with certainty Partial lysis occurred as a late event in these processes, although acute septicaemic salmonellosis is a serious progressive infection in pigs, ordi

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* According to terms in current literature (3), fragmentation means separation of haemoglobin-containing spherules, a process which frequently starts with the formation of buds 'Disintegration is used in a wide sense, covering all the types of destructive processes of red cells described in this paper

narily with lethal outcome. Platelets were frequently trapped in the occlusive material, while leucocytes were only sporadically recognized It seems likely that fibrin at times may also be included, but true fibrin, with its regular cross striation, was not demonstrated within the "thrombi" in this study

Severe stasis is an early step in the development of the "thrombosis". Although recognized for many years, relatively little attention has been paid to the significance of stasis as a cause of tissue necrosis According to *Kreyberg* (24, 25) and *Kreyberg* & *Vermes* (26) true stasis depends on an increased permeability of minute vessels, followed by extensive loss of plasma and haemoconcentration This process is frequently irreversible, resulting in a severe disturbance in the microcirculation with complete cessation in the blood flow, the cells are left dry and tightly packed within vessel luminae The conglutinated cells necrotize, coalesce and may later occlude the vessels as hyaline "thrombi" Thus, the effect of stasis may be equivalent to real microcirculatory thrombosis and cause necrosis in surrounding tissue (24–26)

Experimental GSR is classically induced by two subsequent intravenous injections of bacterial endotoxin, given at 24-hour intervals (18, 33, 46) Most authors have, during recent years, attributed the development of GSR to disseminated intravascular coagulation (18, 33, 34, 42, 45), and the occlusive masses precipitated in glomerular capillaries during the reaction have been interpreted as fibrin or a fibrin like material (5, 41, 45) It seems probable, however, that the ultrastructural composition of the occlusive masses may show some variabilities, possibly dependent on the experimental procedure and the developmental time of the lesions In the present investigation, severe stasis and extensive dilatation of glomerular capillaries and the microcirculation of the skin was an essential feature, the main component of the occlusive material being derived from stagnant, disintegrating red cells, and not from the circulating fibrinogen This view is indirectly supported by the fact that massive heparinization does not modify the autopsy picture in experimentally induced salmonellosis in pigs (38) Furthermore, it seems likely that the material which occludes most elements in the microcirculation in the skin and causes widespread "thrombosis" in the microcirculatory system of other organs, must be present in the blood in very great quantities

Administration of endotoxin is followed by endothelial injury (13, 32, 44, 45), and endothelial lesions and haemodynamic mechanisms (2, 11, 14, 17, 27, 35, 52) have also been pointed out as important factors in the pathogenesis of endotoxin shock and the GSR In the present study, the endothelial changes were quite advanced, but it seems obvious that they had gradually developed as the infection progressed and thus, at least in part, were due to hypoxaemia Therefore, it may be concluded that this material was unfitted for determination of endothelial damage as a possible primary event in the pathogenesis of the GSR It has been suggested, however, that microangiopathy may be causally related to morphological alterations in circulating red cells (6), and it seems probable that microangio-endothelial modifications may also be a significant pathogenetic factor in porcine salmonellosis It has been claimed that endotoxin increases vessel permeability and exogenous application or endogenous release of endotoxin may thus probably lead to haemoconcentration (7, 9, 12, 47–49) It seems likely that the circulatory collapse in porcine salmonellosis largely is brought about by a catastrophic leakage of plasma through microcirculatory vessels It is also obvious that the glomerular capillaries constitute a critical site in the microcirculation, because of the exceptional haemoconcentration which normally occurs in the glomeruli, in connection with the filtration process This phenomenon is clearly emphasized by the fact that the renal lesions of the GSR are prevented by experimental ureteral overpressure or blockade (4, 10, 50, 52), a measure which represses the filtration without

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ULTRASTRUCTURAL PATHOLOGICAL CHANGES IN INTESTINAL SUBMUCOSAL ARTERIOLES IN ANGIOTENSIN-INDUCED ACUTE HYPERTENSION IN RATS

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The ultrastructure of intestinal submucosal arterioles in rats with angiotensin induced hypertension was examined Dilated arteriolar segments showed focally localized severe changes (patches) with gaps in the endothelial cell layer and with discontinuities in the endothelial basement membrane, the internal elastic lamina and the luminal basement membrane of media Through the discontinuities, plasma formed elements of the blood and injected carbon particles penetrated into the arteriolar wall In the patches, severe injury and slighter degenerative changes were observed in all layers of the vascular wall Lesions were most extensive in the media and showed a gradient in severity of changes from the centre to the marginal zone of the patches The observed changes were results of smooth muscle cell necrosis and plasma insudation

The fine structure of the normal intestinal arterioles in the rat and the permeability pathways in segments of these vessels by acute angiotensin induced hypertension are described in the two preceding papers (1974a & b) Examination of semi-thick sections in the light microscope and of thin sections in the electron microscope revealed degenerative changes in the walls of the arteriolar dilatations

The majority of studies concerning the ultrastructure of the hypertensive vascular lesion have mainly described the changes of the intima and in the permeability In only a few studies have the media changes been described in detail, and the problem of the sequence of the changes in the vascular wall has not yet been solved The observations of *Takebayashi* (1970) and *Todd & Friedmann* (1972) indicated that the media is the seat of the primary events In a study of acute angiotensin induced hypertension by *Goldby & Beilin* (1972), the conclusion was that plasma insudation was primary to changes in the media

In the present paper, the fine structural changes of the arteriolar wall following repeated injections of angiotensin were studied

Received 24 iii 74   Accepted 10 vi 74

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MATERIAL AND METHODS

Experimental animals, experimental technique and the fixation procedure are described in earlier

papers (*Thorball* & *Olsen* 1974a & b) 20 dilated and permeable arteriolar segments were examined in the electron microscope

RESULTS

In most of the wall of the dilated segments of the arterioles, the morphological changes were only slight (A), but limited severely damaged areas, in the following called "patches", were observed in all dilated segments (B)

A Changes outside the patches The wall of the dilated segments was reduced by 1 2 5 microns in thickness compared to normal arterioles

The endothelium was thin measuring from 300 Å to 2 4 microns in thickness Its luminal surface was smooth (Fig 4) and the nuclei had no folds in their envelopes (Fig 14) *The endothelial junctions were only slightly* interdigitated and usually appeared S shaped in cross sections of the vessels They were sometimes opened up luminally and/or ablu minally (Fig 14) Small interendothelial gaps without signs of penetration of blood plasma into the vessel wall were seen The cyto plasm contained the usual organelles, but the *caveolae vesicles, filaments and microtubules* appeared more conspicuous than in normal arterioles (Fig 4) The vesicles measured 600 1800 Å in diameter and were more often than in normal arterioles coalesced to form vacuoles In some places, the subendothelial space was slightly dilated

The internal elastic lamina was thin with a thickeness varying from 0 1 to 1 micron The fenestrae in the lamina were larger than in the normal arterioles and measured 0 4 7 *microns in width Their number did not seem* to be increased In a few places, the lamina appeared changed in ultrastructure, being either more dense or less dense than normal The less dense areas were thicker than the remaining part of the lamina

The media (Fig 4 varied from 0 8 to 3 7 microns in thickness The smooth muscle cells appeared stretched The nuclei had a smooth surface and in some places the number of vesicles in the cytoplasm seemed to be increased In a few places, slightly dilated intercellular spaces and muscle cells with a slight ly irregular surface were observed Cyto plasmic vacuoles measuring as much as 2 *microns in diameter were found occasionally* Their content was finely granular or homo-

Fig 1 This survey picture shows the localization of the arterioles examined in this study A dilated arteriolar segment is seen in the submucosa be tween the muscularis mucosa and the muscularis externa Carbon is seen in the arteriolar wall (ar row) C collagen fibres Perfusion fixed Stained with toluidine blue × 200

Fig 2 Schematic drawing of a dilated arteriolar segment showing two "patches in the arteriolar wall and the spread of carbon in the patches (stippled) The patches are oblong with the long axis in the transverse plane of the vessel (two single arrows) The cut patch shows the bulging of the arteriolar wall (double arrow)

Fig 3a Photomicrograph of a transverse section of a dilated arteriolar segment containing two patches merging into each other The central patch area (a), the intermediate zone (b) and the marginal zone (c) are marked Blood cells and platelets are sticking to the arteriolar wall Carbon deposits (CAR) and hyaline like areas are seen in the wall The patches involve 5/6 of the cir *cumference of the vessel The borderline between* the normal looking and the damaged part of the wall is marked by an arrow In the patches the arteriolar wall is much thicker than in the un changed part and thicker than the normal arterio lar wall (compare with Fig 3b) The internal elastic lamina (IEL) is not well defined Perfu sion fixed Stained with toluidine *blue × 1 100*

Fig 3b Transverse section of a normal arteriole from a control experiment The internal elastic lamina (IEL) with many fenestrae (arrows) appears as a stippled dark line Perfusion fixation Stained with toluidine blue × 1,100

Fig 4 This is a part of the same longitudinal section of a dilated arteriolar segment of which another part is shown in Fig 11 The distance betwe n these two parts is 25 microns The arterio lar wall appears normal Many vesicles (ve) and filaments (f) are visible in the endothelium (E) IEL *internal elastic lamina, l lumen, SM* smooth muscle cells, en endothelial nucleus, m mitochondria Perfusion fixation Cacodylate buf fered glutaraldehyde Uranyl block stained × 20 000

muscularis mucosa

C

muscularis externa

1

IEL

a

b

b

CAR

a

b

c

IEL

3a

IEL

IEL

b

2

L

ve

ve

f

E

n

cv

IEL

SM

SM

m

papers (*Thorball & Olsen 1974a & b*) 20 dilated and permeable arteriolar segments were examined in the electron microscope

RESULTS

In most of the wall of the dilated segments of the arterioles, the morphological changes were only slight (A), but limited severely damaged areas, in the following called 'patches", were observed in all dilated segments (B)

A Changes outside the patches The wall of the dilated segments was reduced by 1 2 5 microns in thickness compared to normal arterioles

The endothelium was thin, measuring from 300 Å to 2 4 microns in thickness Its luminal surface was smooth (Fig 4) and the nuclei had no folds in their envelopes (Fig 14) The endothelial junctions were only slightly interdigitated and usually appeared S shaped in cross sections of the vessels They were sometimes opened up luminally and/or abluminally (Fig 14) Small interendothelial gaps without signs of penetration of blood plasma into the vessel wall were seen. The cytoplasm contained the usual organelles, but the caveolae vesicles filaments and microtubules appeared more conspicuous than in normal arterioles (Fig 4) The vesicles measured 600–1800 Å in diameter and were more often than in normal arterioles coalesced to form vacuoles In some places the subendothelial space was slightly dilated

The internal elastic lamina was thin with a thickeness varying from 0 1 to 1 micron The fenestrae in the lamina were larger than in the normal arterioles and measured 0 4 7 microns in width Their number did not seem to be increased In a few places the lamina appeared changed in ultrastructure being either more dense or less dense than normal The less dense areas were thicker than the remaining part of the lamina

The media (Fig 4) varied from 0 8 to 3 7 microns in thickness The smooth muscle cells appeared stretched The nuclei had a smooth surface and in some places the number of vesicles in the cytoplasm seemed to be increased In a few places, slightly dilated intercellular spaces and muscle cells with a slightly irregular surface were observed Cytoplasmic vacuoles measuring as much as 2 microns in diameter were found occasionally Their content was finely granular or homo-

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Fig 1 This survey picture shows the localization of the arterioles examined in this study A dilated arteriolar segment is seen in the submucosa between the muscularis mucosa and the muscularis externa Carbon is seen in the arteriolar wall (arrow) C collagen fibres Perfusion fixed Stained with toluidine blue × 200

Fig 2 Schematic drawing of a dilated arteriolar segment showing two 'patches in the arteriolar wall and the spread of carbon in the patches (stippled) The patches are oblong with the long axis in the transverse plane of the vessel (two single arrows) The cut patch shows the bulging of the arteriolar wall (double arrow)

Fig 3a Photomicrograph of a transverse section of a dilated arteriolar segment containing two patches merging into each other The central patch area (a), the intermediate zone (b) and the marginal zone (c) are marked Blood cells and platelets are sticking to the arteriolar wall Carbon deposits (CAR) and hyaline like areas are seen in the wall The patches involve 5/6 of the circumference of the vessel The borderline between the normal looking and the damaged part of the wall is marked by an arrow In the patches, the arteriolar wall is much thicker than in the unchanged part and thicker than the normal arteriolar wall (compare with Fig 3b) The internal elastic lamina (IEI) is not well defined Perfusion fixed Stained with toluidine blue × 1,100

Fig 3b Transverse section of a normal arteriole from a control experiment The internal elastic lamina (IEL) with many fenestrae (arrows) appears as a stippled dark line Perfusion fixation Stained with toluidine blue × 1,100

Fig 4 This is a part of the same longitudinal section of a dilated arteriolar segment of which another part is shown in Fig 11 The distance between these two parts is 25 microns The arteriolar wall appears normal Many vesicles (ve) and filaments (f) are visible in the endothelium (E) IEL internal elastic lamina, L lumen, SM smooth muscle cells, en endothelial nucleus m mitochondria Perfusion fixation Cacodylate buffered glutaraldehyde Uranyl block stained × 20 000

Fig 5 Schematic drawing of the central part of a patch with an endothelial gap end penetration of plasma, carbon and cellular elements of the blood into the media The media is totally dis rupted with cellular elements, plasma and carbon intermingling The smooth muscle cells (SM) have almost disappeared but a few severely injured remnants are left E endothelial cell, L lumen

Fig 6 Schematic drawing showing the intermedi ate zone of a patch Compartments bounded by basement membanes and containing a homogeneous or finely granulated substance are seen in the media area (M) Carbon particles and remnants of cell membranes are present along the basement membranes E endothelial cell, L lumen

Fig 7 Schematic drawing of the marginal zone of a patch The smooth muscle cells (SM) appear irregular and contain intracellular vacuoles The intercellular space (IS) is increased Similar changes could be found elsewhere in the dilatations without any detectable relation to patches E endothelial cell, L lumen

Fig 8 Photomicrograph of a patch from a longi tudinal section of a dilated arteriolar segment An accumulation of blood platelets (THR) and leukocytes (LEU) adheres to the arteriolar wall Large accumulations of carbon (CAR) and an erythrocyte (RBC) are seen in the wall, which is severe ly damaged Perfusion fixed Stained with toluidine blue × 1,200

Fig 9 Central part of a patch seen in a trans versal section of a dilatation The site of penetra tion into the media is illustrated To the right an endothelial protrusion (EP) and carbon (and plasma) have penetrated into the media through an endothelial gap, a fenestra in the internal elastic lamina (IEL) and a discontinuity of the basement membrane On the left there is a gap (EG) in the endothelium (E) and a fenestra (FEN) in the internal elastic lamina The media is severely injured Unidentified cellular fragments (CF) with a tendency to form myelin like figures (thick arrow), remnants of smooth muscle cells (SM), part of a granulocyte (PLEU) membran ous and fibrillar elements (arrow), clear spaces granular masses and an erythrocyte (RBC) are seen Revealed by serial sectioning the cellular fragment marked EP is an endothelial protrusion Note the discontinuation (double arrow) of the adventitial basement membrane (ABM) EBM endothelial basement membrane, EF endothelial fragments, L lumen SES subendothelial space Immersion fixation Phosphate-buffered glutaraldehyde × 12 000

Fig 10 Central part of a patch as in Fig 9 In the lumen (L) are blood platelets and parts of neu

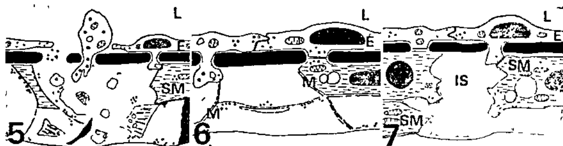
geneous and rather electron dense The adventitia was normal

Colloidal carbon was not found in these parts of the arteriolar wall

B The patches All layers were damaged in the patches, which were oblong in shape with the long axis oriented in the transverse plane of the arteriole, that is to say parallel to the muscle cells of the media (Fig 2) The pat ches covered 1/10 to 5/6 of the vessel circumference which measured from 280 to 420 microns (Fig 3a) Their width in sections cut longitudinally through the arteri oles was from 20 to 60 microns (Fig 8) Corresponding to the patches, the vessels were bulging (Fig 2) and the wall had increased by 1 to 4 microns in thickness in relation to the rest of the dilatation (Figs 3a 11 14) and perhaps a little in relation to the normal arteriole (Fig 3b) The damages in the patches included degeneration of the cells disorganization of the structures form ing the wall and penetration into the wall of carbon plasma and formed elements from the blood stream The extent of the spread of carbon particles was a little less than that of the patches (Fig 2) The most pronounced changes occurred in the centre of the patches where also the leakage ways were found and from here the changes decreased towards the periphery The endothelium and the internal elastic lamina showed changes which were qualitatively as well as quanti

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trophilic granulocytes The endothelium (E) appears as small fragments (EF) some of which are vacuolated and ruptured Thick arrow marks place of penetration Thin arrow marks the left edge of the endothelial gap Several fenestrae are seen in the internal elastic lamina (IEL), which has changed in ultrastructure In the media a mess of remnants of smooth muscle cells (SM) extracellular myofilaments (f) and mitochondria (m) granular areas (GRAN) blood platelets (THR) and parts of leucocytes (LEU) Carbon has spread throughout the media The adventitial basement membrane (ABM) appears intact via vacuoles Perfusion fixation Cacodylate-buffered glutaraldehyde Uranyl block stained. × 8,100


L
E
SM
5
L
E
M
M
6
L
E
SM
IS
SM
7
L
THR
LEU
CAR
CAR
RBC
EBM
EG
SES
L
E
IEL
IEL
EP
CF
CF
SM
9
ABM


L
va
va
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E
EP
EF
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IEL
IEL
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GRAN
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THB
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ABM
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SM
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Figs 9 and 10 show the severe changes in this area At one or more places, coincident discontinuities in the intima, the internal elastic lamina and the adjoining basement membrane of the media had allowed carbon, plasma and cellular elements of the blood to penetrate into the media The media itself is completely disintegrated with deposits of carbon and plasma In most cases, the adventitial basement membrane is intact, hindering carbon and plasma from penetrating into the adventitia

The smooth muscle cells are irregular, often condensed, and with only a few or no organelles (Figs 9, 10, 11) Often the myofilaments appear as thick and thin elements (Fig 13) Some muscle cells are ruptured, and in these cases carbon may be found intracellularly (Figs 11, 15) Other cellular elements which can be recognized are more less altered endothelial protrusions (Fig 9), leucocytes (Figs 10, 16), thrombocytes (Fig 10) and erythrocytes (Fig 9)

The extracellular spaces are greatly expanded and contain remnants of cellular membranes, vacuole-like structures, mitochondria, fibrillary material (which may be myofilaments or loose fibrillary fibrin), carbon particles and granular masses of varying density, probably representing penetrated plasma (Figs 9, 10, 11, 16)

In a few cases at the point of penetration, the luminal smooth muscle cell appeared relatively intact, but was displaced by a "lake" of plasma, from which the plasma apparently had penetrated further into the media (Fig 15) The extent of the central area was 10–30 microns in transversal sections of the arterioles

b) *The intermediate zone of the patch* (Fig 6) This zone measured 10–50 microns in width Most of the media area appeared as finely granular masses of varying electron density in compartments surrounded by basement membranes along which remnants of cell membranes from smooth muscle cells were found (Fig 14) Carbon also occurred along the basement membranes especially the adventitial one The muscle cells were in most cases condensed and irregular, contained thick and thin myofilaments and were often located in the centre of the compartments Extracellularly located cellular remnants and other extracellular components as described in the central patch area were also seen in the compartments (Figs 14, 17)

In many cases, the changes involved only the adventitial part of the media, the luminal muscle cells being normal or nearly normal

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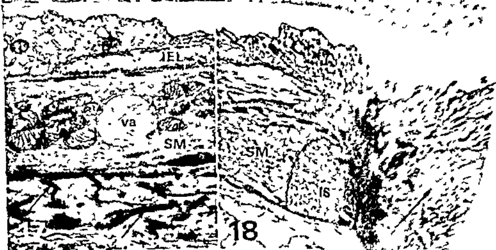
Fig 15 Transversal section of a dilation In the middle of the picture, the right edge (thick arrow) of an endothelial gap is seen The gap is almost totally occluded by parts of mononuclear cells (MON) Fenestrae (FEN) of the internal elastic lamina (IEL) are seen In the media, the luminal smooth muscle cell (SM) is dislocated by an accumulation of insudated plasma (INS) Abluminal to this cell, areas with plasma insudation (INS) and injured smooth muscle cells (SM') are seen cm cell membrane of smooth muscle cell, CAR carbon Perfusion fixation Cacodylate buffered glutaraldehyde Uranyl block stained × 11,800

Fig 16 The central part of a patch is seen The endothelium (E) is intact and very thin In the media is a mononuclear cell (MON) in which a small amount of carbon is found intracellularly (thick arrow) Fibrillar structures (arrows), cellular fragments (CF) and carbon (CAR) surround the cell IEL internal elastic lamina, L lumen Immersion fixation Phosphate buffered glutaraldehyde × 15,000

Fig 17 The intermediate zone of a patch is seen The internal elastic lamina (IEL) is of very low density The luminal smooth muscle cell (SM) contains a large vacuole (va) In the adventitial layer of media, amorphous material and dense fibrillar bundles (arrows) are seen Perfusion fixation Cacodylate-buffered glutaraldehyde Uranyl block stained × 13,000

Fig 18 The marginal zone of a patch A junction in the endothelium (E) is slightly opened up luminally and abluminally (EJ) A small endothelial protrusion (EP) forming a myoendothelial junction (arrow heads) is seen The intercellular space (IS) of the media is dilated, containing very fine granular material The muscle cells (SM) have an irregular surface and in the cell at right a big vacuole like structure (arrow) with very fine granulated content is seen Perfusion fixation Phosphate buffered glutaraldehyde × 11,000

MON
MON
FEN
IEL
INS
SM
16



(Fig 17) This was more pronounced the greater the distance from the centre of the patches With the aid of serial sections, it was found that in such cases the spread of plasma from the central area mainly took place in the adventitial layer of the media

c) *The marginal zone of the patch* (Fig 7) This zone had dilated intercellular spaces measuring up to two microns and containing a homogeneous, granular material, in a few cases with carbon particles (Fig 17) The smooth muscle cells were irregular in form and very often contained irregular vesicles and vacuoles The vacuoles measured up to 1 micron in diameter and contained an amorphous or finely granular material (Figs 17, 18) Furthermore, the muscle cells could vary in electron density and in number of organelles compared with normal muscle cells

In the patches *the adventitia* was normal apart from occasional discontinuities of the adventitial basement membrane of the media (Fig 9)

DISCUSSION

An important finding in the present study is the occurrence of patchy injured areas in the wall of dilated arteriolar segments The injury is most severe in the media and connected with penetration of plasma into the arteriolar wall through gaps in the endothelium, dilated fenestrae in the internal elastic lamina and discontinuities in the basement membranes

The sporadic finding of erythrocytes and the totally disrupted media in the central area of the patches indicate a high pressure of the insudating plasma

In the same areas thrombocytes and leucocytes were found Leucocytes were seen more frequently than erythrocytes indicating an active migration of the leucocytes (for literature see *Olsen* 1971 *Hatt* 1972)

The extent of the finely granulated or homogeneous areas and the increased thickness of the arteriolar wall corresponding to the patches shows that plasma spreads through the whole patch As also observed by *Goldby* & *Beilin* (1972 the spread takes place mainly in the adventitial layer of the media

Patchy localization of the changes caused by the angiotensin induced hypertension was observed by *Goldby* & *Beilin* (1972) These authors interpreted the changes of media except those in the central patch area as results of dislocation due to plasma insudation In the present work, however, degenerating and necrotic muscle cells were seen in the entire patch In the intermediate zone the damage was severe with rupture of plasma membranes Thick myofilaments, which were very seldom found in the normal arteriole (1974a), were observed in these cells It is difficult to explain the increase in number of thick filaments, but such filaments may appear in connection with (abnormal?) contraction (for literature see *Devine* & *Somlyo* 1971) Many of the abnormal muscle cells in the patches are small, irregular and condensed especially in the intermediate zone They are separated from their basement membranes and from fragments of their plasma membranes by large spaces (Fig 14) This situation could arise either from a rigorous contraction of the smooth muscle cells or be provoked by plasma penetrating under high pressure

Most of the observed changes of the media seem more easily explained by violent contraction of the muscle cells than by dislocations Observations clearly indicating dislocation from penetrating plasma were in fact very rare (Fig 15)

The electron microscope used in the present study was made available by a grant (A 1/65) to Dr *H Moe* from *the Danish Medical Research Council* and the study was further supported by a grant to Dr *J Bing* from *King Christian X Foundation* and by a grant from the *Danish Heart Foundation*

The authors are very grateful to Dr *Harald Moe* and Dr *Jens Bing* for reading and criticizing the manuscript We wish to thank Dr *Jorgen Fog gaard* for helpful discussions and Miss *Kirsten Sjoberg* for technical assistance

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ADDENDUM

After the present study was accepted for publication the authors attention was drawn to a recent study by *Wiener & Giacomelli* (Am J Path *72* 221–231, 1973) They examined mesenteric vasculature after infusion of angiotensin for 2 to 4 hours and found a sequence in the changes depending on the duration of the angiotensin infusion The injury of the vascular wall started with contraction of the smooth muscle cells of media Gradually degeneration and necrosis of the media was developed and not until then carbon and blood constituents were observed in the media This very interesting observation is inconsistent with the results of *Goldby & Beilin* (1972) They did not find essential difference between the changes observed after angiotensin infusion during 1 hour and during 4 hours Furthermore *Giese* (Acta path microbiol scand *62* 497 515, 1964) found penetration of carbon into the arteriolar wall already from 50 seconds to 7 minutes after the beginning of infusion of angiotensin

(Fig 17) This was more pronounced the greater the distance from the centre of the patches With the aid of serial sections, it was found that in such cases the spread of plasma from the central area mainly took place in the adventitial layer of the media

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The authors are very grateful to Dr *Harald* ...*berg* for technical assistance

The patients represent a wide clinical spectrum of disease,—the majority of mild cases being seen among out patients According to the course of disease, 64 cases were of the chronic intermittent type, 22 of the chronic continous type and 2 were fulminating cases Nine cases could not be classified

Percutaneous liver biopsy was performed in all patients, using the Menghini technique The biopsies were fixed in formalin and stained by haematoxylin and eosin

The presence of serum antibodies against colon mucosa epithelial components was detected by means of an indirect immunofluorescence technique Colon tissue was obtained from monkey (guenon, *Cercopithecus aethiops*) The tissue was cut into approximately 1 × 1 × 1 cm blocks, were rapidly frozen to –70° C in acetone solid CO_2 mixture and stored at 20° C until use The blocks were cut into sections of 4 μm in a Pearse cryostat at –20° C, fixed in 95 per cent ethanol for 10 minutes at 4° C, mounted on individual slides, air dried for 20 minutes and used in the immunofluorescence studies within hours The sections were incubated with undiluted serum at room temperature in moist chambers for 30 minutes, washed carefully for 20 minutes with a Coon s buffer in great surplus The sections were then incubated with a fluorescence isothiocyanate conjugated polyvalent antiserum of horse origin to human gammaglobulins (Borrughs Wellcome & Co) in a 1 10 dilution and kept at room temperature in moist chambers for 30 minutes Finally they were washed for another 20 minutes with buffer as described above The slides were mounted with a drop of 10 per cent glycerole in saline under a cover slip and examined within 2 3 hours in an immunofluorescence microscope (Leitz Orthoplan with HBO 200 W high vacuum burner, DG 38 and BG 12 exiter filters, dark ground condenser and 530 μm barrier filter) The results were read blindly by two independent readers and recorded semiquantitatively + +, +, +/– or negative Only sera read as + + or + in three individual experiments were recorded as positive This technique and the serological evaluation has been described in detail by *Marcussen & Nerup* (1973)

RESULTS

Thirty nine patients showed histological liver changes Six types of histological changes could be indentified (Table 1) 1) Very slight mostly diffuse cellular infiltration without biliary ductular and periportal involvement was seen in 14 specimens 2) Slight to moderate cellular infiltration with biliary

TABLE 1 *Liver Biopsy in 97 Patients with Ulcerative Colitis Histological Results*

Normal		58
Mild degree of inflammation	14 (8)	
Periportal inflammation	20 (20)	
Chronic pericholangitis	3 (3)	
Cirrhosis	1 (1)	
Focal necrosis	1 (0)	
Fatty change	7 (7)	
Number of abnormal specimens	46	
Number of patients with abnormal histology		39
Total		97

If more than one histological type was present in individual patients, the dominating finding is shown in brackets

ductular involvement predominantly consisting of plasma cells, lymphocytes, histiocytes and macrophages, the polymorphonuclear leucocytes forming a minority of the total cells (=portal triaditis) in 20 specimens 3) Fibroblastic proliferating and ductal fibrosis was registered in three cases, 4) post necrotic cirrhosis in one and 5) focal necrosis as the only manifestation in one 6) Moderate (<20 per cent cells involved) degeneration with fat droplets was found in 5 cases,—and slight degree (<10 per cent) in two patients In 7 patients, two types of histological changes occurred

Nine patients (19 per cent) showed anti colon circulating antibodies (Table 2) This frequency does not differ from our previous findings of 20 per cent circulating antibodies in u c (*Marcussen & Nerup* 1973) (95 per cent confidence limits, 14–28 per cent) in 145 patients and is in accordance with findings obtained by other groups (*Harrison* 1965, *Wright & Truelove* 1966, *McGiven et al* 1967) In 7 of the 9 patients with circulating antibodies abnormal liver histology was found, including mild cell infiltration in 1 case, periportal inflammation in 5 and cirrhosis in 1 Using the described histological parameters of liver disorder, no correlation with the presence of anti colon antibodies was seen ($0\ 40<p<0\ 45$)

TABLE 2 *Abnormal Liver Histology and Circulating Serum Antibodies against Colon Mucosa in 97 Patients with Ulcerative Colitis*

	Number of patients	Antibody negative	positive	per cent
Abnormal liver histology	39	32	7	(18)
Normal liver histology	58	46	12	(21)
Total	97	78	19	(19)

DISCUSSION

Histological liver changes were shown in 39 patients (=40 per cent, 95 per cent confidence limits, 30–51 per cent) Slight cellular infiltration was found in 8 patients, whereas pronounced histological changes were observed in 31 The dominating feature was inflammation in and around the portal tracts This is considered to be the most common liver lesion in u c (*Mistilis* & *Goulston* 1965)

The aetiology of liver disorder in u c has been proposed to be portal bacteraemia and antibody formation against the bowel flora (*Boden et al* 1959) *Koffler et al* (1962), using an immunofluorescence test, demonstrated a common antibody against colonic mucosa and hepatic biliary ductuli in some patients with u c In the present series, 23 cases of varying degree of cholangitis were noted, but only 5 of these patients (=22 per cent) had antibodies in the blood, i e the same occurrence in the cholangitis group as in the total patient group Thus the present study gives no support to the above mentioned theory

Fatty infiltration was noted in 7 of the patients This seems to be in contrast to reports claiming that this is the most frequent histological finding (*Monto* 1959) Fatty liver in u c appears to be caused by nutritional deficiencies in severe long standing cases (*Sherlo* 1968) and accordingly, it was a more common finding in earlier, postmortem studies *Monto* 1959)

Cirrhosis was found in 1 patient The association with u c has often been mentioned in case reports The frequency varies from less than 2 to more than 15 per cent, mostly about 3 per cent (*Perrett et al* 1971) Usually the type is post necrotic (*Mistilis et al* 1965)

Several observations raise the question whether liver disease as an extra intestinal manifestation of u c should be regarded as a complication (*Anthonisen et al* 1966 *Wright* & *Truelove* 1966) or an associated condition—especially as an immunological phenomenon *Gray et al* (1958) suggested the latter connection on the basis of 8 cases with systemic lesions, positive LE cell test and/or complement fixation test *Broberger* & *Perlmann* (1959) have supported this concept by experimental studies, and studies by *Broberger* (1961) and *Harrison* (1965) have supported the auto immune theory *Perrett et al* (1971) suggested that the fact that the greater part of positive immunological tests was seen among patients with u c and chronic hepatitis may indicate that multiple organs are involved and, because of tissue damage, an increased release of tissue antigens with resulting antibody formation takes place

The presence of circulating anti colon antibodies in u c is not associated with severity, extent and extra-colonic manifestations in u c *Harrison* 1965, *Marcussen* 1974) This is in accordance with Perrett et al (1971) who examined patients with u c for the occurrence of anti colon antibodies, several organ related antibodies, antinuclear factor and levels of immunoglobulin, and found that, apart from high values of IgG, none of these parameters showed a positive

correlation to the presence of systemic complications of u c.

Recently *Triger et al* (1972) and *Bjorneboe et al* (1972) demonstrated positive agglutinating antibody titres against antigens of intestinal origin and of colon of rats in patients with severe hepatic disease This was taken in support of a theory of insufficiency in liver patients—especially in cirrhotic patients—to sequestrate intestinal antigens The antigens bypassing the diseased liver after absorption from the gut were [illegible] to [illegible] by [illegible] ing intestinal antibodies to bacterial or viral antigens [illegible] co existing liver involvement (*Triger et al* 1972) That the present study fails to demonstrate a correlation between liver disorder and circulating anti colon antibodies speaks against the "mal sequestrating" theory Differences in immunological techniques and types of liver disorders examined in the various studies do not justify a direct comparison An identical frequency of agglutinating intestinal antibodies in liver patients with different stages of fatty changes or cirrhosis was found by *Prytz et al* (1973), which suggests that factors other than severity of disease influence the ability to develop antibodies

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This study was supported by *The Danish Medical Research Council* and *"Peter Laurids Pedersens legat til støtte for lægevidenskabelig forskning"*

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Acta path microbiol scand Sect A, 82 719–729, 1974

INVASIVE BREAST CARCINOMA WITH LOBULAR INVOLVEMENT

Frequency and Location of Lobular Carcinoma in situ

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This paper demonstrates lobular involvement of two morphological different types Lobular carcinoma in situ and carcinomatous extension to the lobule in 70 non selected cases of invasive breast carcinoma Carcinomatous involvement of the lobules was found in 34 cases In 16 of these cases, it was not possible to distinguish the lobular changes morphologically from lobular carcinoma in situ whereas, in 13 cases, carcinomatous extension to the lobules was found In 5 cases the type of carcinomatous involvement of the lobules could not be established with certainty In the 16 cases with lobular carcinoma in situ, 5 of the lesions were found at the edge of the invasive carcinoma, 4 were completely outside the tumour area, and in 7 cases the lesions were found both at the edge and completely outside the tumour area In 59 per cent of cases the lesions were multicentric Thirteen of the 16 cases presented lobular carcinoma in situ also in the upper quadrants The diameter of the tumour was not related to the occurrence of lobular carcinoma in situ or to carcinomatous extension to the lobules On the other hand, lobular carcinoma in situ was most frequent in highly differentiated carcinomas A more detailed study of the biology of lobular carcinoma in situ, associated with invasive breast carcinoma, is considered necessary

In 72 out of 73 cases of invasive lobular breast carcinoma, *Newmann* (1966) found remnants of lobular carcinoma in situ In other types of invasive breast carcinoma however, the appearance of lobular carcinoma in situ is considered a rare phenomenon (*Haagensen* 1971) Particularly in connection with intraductal carcinomas, carcinomatous lobular involvement has been mentioned briefly by many authors (e g *Fraser* 1927, *Charteris* 1930 *Cheatle* & *Cutler* 1931 *Muir* 1941 *Foote* & *Stewart* 1946, *Stapley* & *Dockerty* 1953 *Ozzello* 1959) Out of 435 carcinomas in situ *Farrow* (1970) found 207 lobular types, 147 ductogenic, and 30 which were both lobular and ductogenic He counted the last group together with lobular carcinomas because of concordant histological findings He also found in this last group, as for purely lobular carcinomas in situ, a simultaneous bilateral occurrence of approximately 12 per cent, as opposed to about 1 per cent for ductogenic carcinomas *Fechner* (1971) was the first to make a more systematic study of lobular involvement in cases of invasive carcinoma He demonstrated 45 cases of lobular involvement among 205 cases of invasive ductogenic carcinoma, but in none of the cases was lobular carcinoma in situ observed

Donegan & *Perez-Mesa* (1972) found 36 cases of lobular carcinoma among 1210 patients with malignancies of the breast Most

Received 29 iv 74 Accepted 20 vi 74

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TABLE 1 *Correlation between Important Histological Parameters for Lobules and Various Types of Lobular Involvement in 34 Cases of Invasive Breast Carcinoma*

	With lobular carcinoma in situ n = 16	With carcinomatous extension to lobules n = 13	With uncertain type of lobular involvement n = 5
Involvement of terminal lactiferous duct	9	10	5
Uniform large lobular ductules	15	3	3
Remnants of lobular ductules	0	8	0
Loose intralobular connective tissue	16	12	4
Nuclei almost uniform	16	0	4
non uniform	0	13	1
corresponding to invasive carcinoma	1	13	1
mitoses	1	10	3
Cytoplasm pale and vacuolized	11	2	1
eosinophilic and granulated	5	11	4
Reduced cellular cohesion	11	4	4
Necrosis	0	8	2

TABLE 2 *Histological Type of Invasive Breast Carcinoma in 70 Cases*

	With lobular carcinoma in situ n = 16	With carcinomatous extension to lobules n = 13	With uncertain type of lobular involvement n = 5	Total n = 70
Infiltrating carcinoma (C II)	12	13	4	56
Infiltrating and lobular carcinoma (C II III e)	2	0	1	4
Lobular carcinoma (C III e)	1	0	0	2
Medullary carcinoma (C III a)	1	0	0	3
Mucous carcinoma (C III d)	0	0	0	4
Carcinosarcoma (E)	0	0	0	1

TABLE 3 *H[illegible]gical Grading of 57 Cases of Invasive Ductal Breast Carcinoma and the Occurance of Lobular Involvement*

Histological grad	With lobular carcinoma in situ n = 12	With carcinomatous extension to lobules n = 13	With uncertain type of lobular involvement n = 4	Total n = 57
I	6	1	1	15
II	4	6	3	30
III	[illegible]	6	0	12

of them were recognized as a result of increased diagnostic efforts among 77 patients Among them 19, or 24 7 per cent, were found to have lobular carcinoma in the breast alone or in combination with other forms of cancer

During a previous study (*Andersen* 1974), I found 18 cases of invasive breast carcinoma out of 52 cases of lobular carcinoma in situ Thirteen of these cases had lobular carcinoma in situ simultaneously with the invasive carcinoma Four of these were ductogenic, whilst the remaining 9 were lobular In none of these cases was it possible to distinguish the lobular involvement from lobular carcinoma in situ

Similar to the investigations made by *Farrow* (1970) and *Donegan & Perez Mesa* (1972), the above study indicates that the frequency of lobular carcinoma in situ appearing concurrently with invasive breast carcinoma might be higher than generally assumed

Since the consequence of finding lobular carcinoma in situ is today most often ipsilateral mastectomy associated with simultaneous biopsy of the contralateral breast, or even primary bilateral mastectomy (*Benfield et al* 1972), it is important, apart from purely theoretical considerations, to recognize the frequency of lobular carcinoma in situ with simultaneous invasive breast carcinoma and, in particular, the bilateral occurrence of lobular carcinoma in situ and/or invasive breast carcinoma simultaneous as well as non-simultaneous During the present study I have made an attempt to assess the frequency and the location of lobular carcinoma in situ concurrent with invasive breast carcinoma

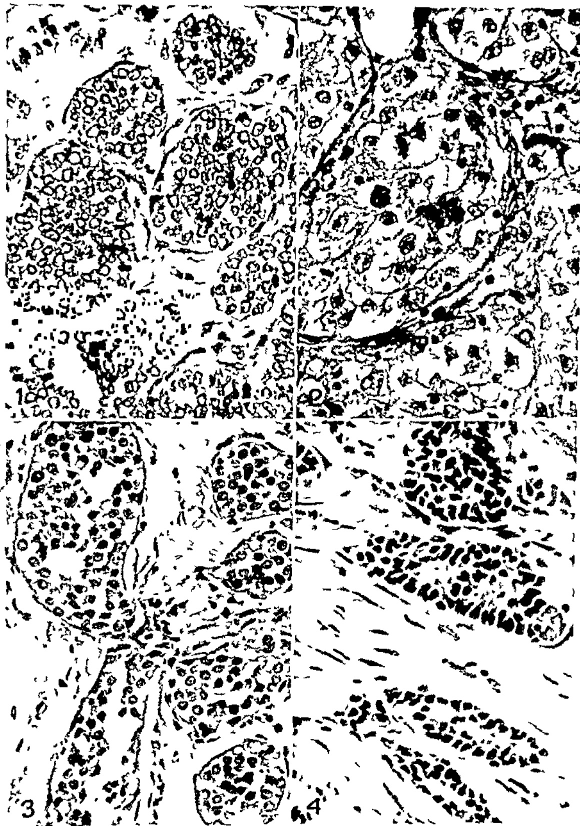
MATERIAL AND METHODS

The material in the present study consisted of non selected mastectomy specimens from 70 women with invasive bresat carcinoma The series originates from the Institute of Pathology at the Copenhagen Municipal Hospital During surgery, the mastectomy specimen was marked and the site is checked by a review of the clinical case records Following complete fixation, close sections were made from the breast parenchyma and, if the size of the tumour so permitted, at least 4 large tissue sections were obtained, particularly from the marginal zone In all cases large sections were taken also from all quadrants between 4 cm and 6 cm from the nipple and furthermore, sections from any pathological areas A total of 628 tissue sections were obtained, i e, 9 (6–16) per breast The sections were embedded in paraffin and slides were produced and stained with haematoxylin eosin and van Giesson Hansen, respectively The malignant tumours were classified and graded in accordance with the WHO Classification (1968) It was ascertained whether carcinomatous lobular involvement was present and, if so, the location of such changes were classified as follows

1) in the tumour and/or at its edge,
2) in other sites of the tumour quadrant,
3) in other quadrants

Carcinomatous lobular involvement was assessed according to the parameters shown in Table 1 In this connection it was assessed whether a certain case was lobular carcinoma in situ and/or carcinomatous lobular extension The criteria for lobular carcinoma in situ were the same as those previously described (*Andersen* 1974) The following criteria support the evidence of lobular carcinoma in situ and, at the same time, contradict carcinomatous extension uniform cytological appearance unlike the concurrent invasive carcinoma, no, or only few, mitoses, no necrosis, uniform affection and size of ductules On the other hand, the lack of affection of terminal lactiferous ducts cannot be used as a criterion for lobular carcinoma in situ, as stated by *Fechner* (1971), because the terminal lactiferous ducts will often be involved also in lobular carcinoma in situ (*Antonius & Jones* 1963, *Haagensen* 1971, *Andersen* 1974) In primary presumed cases of lobular carcinoma in situ it has been assessed, if necessary by stepwise sectioning whether a direct ductal connection existed between the invasive carcinoma and the lobules involved In such cases the changes were not classified as lobular carcinoma in situ, but as uncertain lobular involvement This group comprises cases in which the carcinomatous lobular involvement could be classified neither as lobular carcinoma in situ nor as carcinomatous extension

Finally, it was assessed whether the following parameters were present or not, as well as the possible site of such parameters in relation to the invasive carcinoma, as stated above proliferation of duct epithelium macrocysts, microcysts, apocrine metaplasia, localized adenosis, blunt duct adenosis, papillomatosis, tendency to fibroadenoma, fibroadenoma, papilloma, ductectasia


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RESULTS

Carcinomatous involvement of the lobules was found *in 34 cases (48 per cent)* In 16 cases the findings complied with the criteria for lobular carcinoma in situ, whereas the changes were classified as carcinomatous extension in 13 cases The remaining 5 cases were included in the group of uncertain lobular involvement In 5 of the 16 cases with lobular carcinoma in situ there were also a few lobules with carcinomatous extension The distribution of the various lobular parameters in the 3 groups is shown in Table 1 In the 3 cases of carcinomatous lobular extension without involvement of the terminal lactiferous ducts, however, the extension was most likely of a stromal nature with secondary invasion in the ductules The remnants of the original ductules were often small and insignificant, but they were surrounded by a thick eosinophilic membrane (haematoxylin-eosin) which was a fair clue Amongst the 16 cases of lobular carcinoma in situ the nuclei were fairly small and uniform in 7 cases (Type A), in 6 cases they were larger and slightly varied (Type B), and in 3 cases lobules presenting both Types A and B changes were found Examples of all three groups of lobular involvement are shown in Figs 1–12 The types of invasive carcinoma in cases with lobular carcinoma in situ, carcinomatous lobular extension, uncertain lobular involvement and in the entire material are presented in Table 2 The relationship between the same groups and the differentiation of the carcinomas as regards the purely ductogenic carcinomas, is shown in Table 3 Hence, lobular carcinoma in situ seemed to be more frequent in highly differentiated invasive carcinoma, contrary to carcinomatous extension, but this was not found significant ($P=0\ 10$ by correlating grade I cases against grade II and III cases)

In the *16 cases with lobular carcinoma in* situ the changes in 4 cases were found only outside the area of the invasive carcinoma, although in one case it was found concurrently in the tumour quadrant In 12 cases the lobular carcinoma in situ was found at the edge of the invasive carcinoma and in 7 cases also outside this area 2 in the tumour quadrant, 2 in the other quadrants and in 3 cases both in the tumour quadrant and in the remaining quadrants Hence lobular carcinoma in situ outside the area of the invasive carcinoma was found in 11 cases In 9 of the 16 cases with lobular carcinoma in situ, multiple areas (quadrants) with these changes were found, whereas lobular carcinoma in situ was localized to one area (quadrant) in the remaining 7 cases In only *2 cases was lobular carcinoma in situ localized* to one lobule, whereas more lobules were involved in the remaining cases The frequency of involvement of the various quadrants in the 16 cases of lobular carcinoma in situ was as follows upper outer quadrant, 11 cases upper inner, 6, lower outer, 8, lower inner, 3, central, 1 In 13 of the 16 cases the upper quadrants were also affected

No correlation was found between size of tumour and apperance of lobular carcinoma in situ or carcinomatous lobular extension

Fig 1 45 year-old female (7118/69) with medullary carcinoma (C III a) Section of one of many lobules with carcinoma in situ Note the smooth monotonous appearance This is due both to uniform large ductules and to uniform proliferating epithelial cells, equally distributed in the ductules without stratification Haematoxylin eosin 456 ×

*Fig 2 Same case as that shown in Fig 1 For pur*poses of comparison, a section is shown of the invasive carcinoma, presenting a completely different cytological picture from that seen in the lobular carcinoma in situ Haematoxylin-eosin 456 ×

Fig 3 52 year old female (6926/69) with invasive ductogenic carcinoma (C II) Section of one of many lobules with lobular carcinoma in situ, selected to illustrate the lower diagnostic limit applied Haematoxylin eosin 456 ×

Fig 4 Same case as that shown in Fig 3 Section of highly differentiated invasive carcinoma In this case the cytological difference between the lobular carcinoma in situ and the invasive carcinoma is slight Haematoxylin-eosin 456

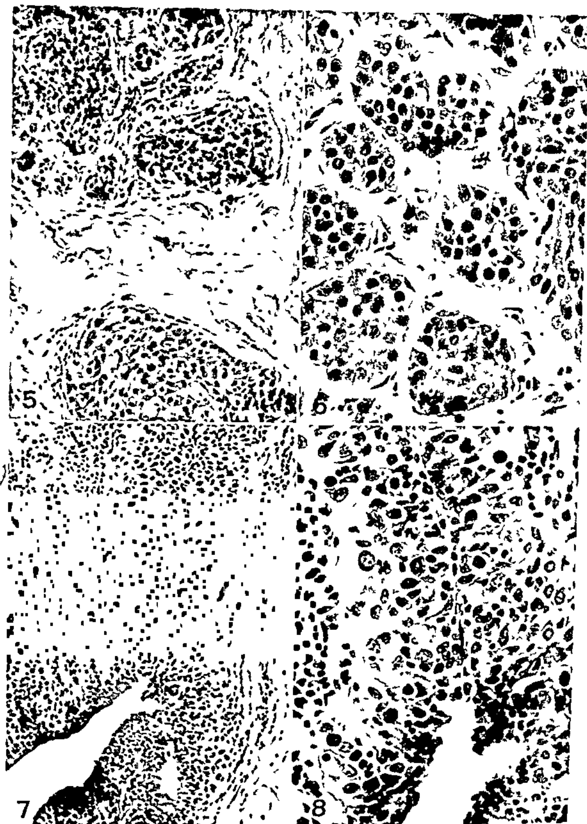

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TABLE 4 *Age Distribution in 16 Cases with Lobular Carcinoma in situ and Simultaneous Invasive Breast Carcinoma*

30–39 years	40 49 years	50 59 years	60–69 years	70–79 years	80 89 years	90 99 years	Total
0	3	3	5	3	2	0	16

TABLE 5 *Age Distribution in 13 Cases of Invasive Breast Carcinoma with Extension to Lobular Ductules*

30–39 years	40 49 years	50 59 years	60–69 years	70–79 years	80 89 years	90–99 years	Total
1	4	3	2	3	0	0	13

TABLE 6 *Age Distribution in 36 Cases of Invasive Breast Carcinoma without Lobular Involvement*

30–39 years	40–49 years	50 59 years	60 69 years	70–79 years	80 89 years	90–99 years	Total
1	3	7	13	7	3	2	36

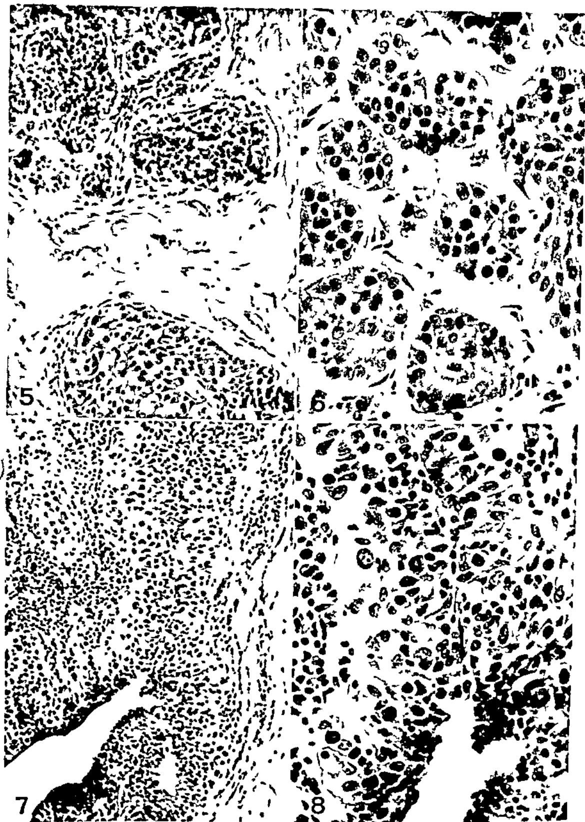
Fig 5 62 year old female (4819/69) Sections of lobule and terminal lactiferous duct (lower part of the figure) The terminal lactiferous duct presents intraductal carcinoma and the lobule presents massive epithelial proliferation (see below) Haematoxylin eosin 182 ×

Fig 6 Same case as Fig 5 The lobular changes correspond to lobular carcinoma in situ, but because of the direct continuity between the ductal carcinoma and this lobule the case is classified as lobular involvement of uncertain type Haematoxylin-eosin 456 ×

Figs 7 and 8 77 year-old female (4685/69) Section of longitudinally cut lobule with normal terminal lactiferous duct in the lower part The ductules present massive epithelial proliferation with pronounced polymorphism of the nuclei and many mitoses In spite of the lack of continuity between the ductal carcinoma and this lobule it is nevertheless classified as lobular involvement of uncertain type and not as lobular carcinoma in situ because of the extremely polymorphic appearance Note also the severe inflammatory reaction with plasma cells and lymphocytes This is not characteristic for lobular carcinoma in situ Haematoxylin-eosin 182 × (Fig 7) and 456 × (Fig 8)

The average age of the patients included in the present study was 62 years (range 31 91 years) As regards lobular carcinoma in situ, the average age was 63 years, and for carcinomatous lobular extension it was 56 years, in cases with uncertain lobular involvement 60 years, and, in the remaining cases without any lobular involvement, the average age was 65 years If the 4 cases with mucous carcinoma and the one with carcinosarcoma were excluded from the last group, the average age in this group did not differ from that found in the entire material The age distribution is shown in Tables 4–6

As regards the parameters which, apart from fibrosis and non specific inflammation, are included in the concept of fibroadenomatosis and fibroadenoma, papilloma, and duct-ectasia their frequency is shown in Table 7 Hence the qualitative appearance does not differ significantly in the various groups Neither was there any qualitative difference in the appearance of these parameters between the tumour quadrant (or quadrants)


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condition deserves further investigation It is suggestive, however, that among the 30 patients of *Haagensen* (1971) with a wide variety of types of breast carcinomas and simultaneous lobular carcinoma in situ, a total of 39 invasive breast carcinomas developed

Out of 10 of the 45 cases with carcinomatous extension, *Fechner* (1971) found duct carcinoma in the contralateral breast in 2 cases, and in one of these, lobular involvement He concluded that this frequency of lobular involvement is lower than that found in cases of lobular carcinoma in situ and that it is important, therefore, to distinguish between lobular carcinoma in situ and carcinomatous lobular extension I agree that it is essential to distinguish between these two highly different lesions but, apart from this, I do not find that part of his study on bilateral occurrence of lobular involvement to be conclusive, firstly because of the size of the material, and secondly because of the short-term follow-up period

In the present series the multicentric appearance of lobular carcinoma in situ is 59 per cent, which is in good agreement with other investigations On the basis of the literature available, *Warren* (1969) summarized the multicentric appearance to 70 per cent, and, in his comprehensive series of cases of lobular carcinoma in situ and intraductal carcinoma, *Farrow* (1970) found frequencies of 63 per cent and 47 per cent, respectively

The site of lobular carcinoma in situ found in the present study corresponds to the findings made by *Antonius & Jones* (1963) and *Lambird & Shelley* (1969) Lobular carcinoma in situ is most often located in the upper quadrants but may be found throughout all quadrants

Also in cases of lobular carcinoma in situ, the average age in the present series is very high On the other hand, lobular carcinoma in situ without concurrent invasive carcinoma is found most often during the 4th decade but also postmenopausally (*Warren* 1969 *Farrow* 1970, *Andersen* 1974 *Haagensen* (1971) found that only 3 out of 63 patient with lobular carcinoma in situ and simultaneous invasive carcinoma were in the postmenopausal period, but both *Neuman* (1966) and *Fechner* (1972) found severt cases in elderly patients, *Neuman* (1966) found that 8 out of 73 patients were 65 years old or more

The present study gives rise to the following questions is the demonstration of lobular carcinoma in situ simultaneous with invasive carcinoma tantamount to (1) increased frequency of lobular carcinoma in situ in the contralateral breast? (2) increased frequency of invasive carcinoma in the contralateral breast?

The results of the study again bring up the question as to whether invasive breast carcinoma does not, perhaps more than commonly presumed, originate from the lobules and/or the immediately adjacent part of the terminal lactiferous duct (*Dawson* 1933, *Geschikter* 1945) Fusion of the ductules in connection with epithelial proliferation is similar to that often seen in histological specimens Such epithelial formations will often be wrongly classified as ductogenic because of their size, but further study involving in particular the basal membrane and the myoepithelial cells, can often produce evidence that this it not the case *Wellings & Jensen* (1973) reached a similar hypothesis by a quantitative, three dimensional subgross method

CONCLUSIONS

1 The frequency of lobular carcinoma in situ concurrent with invasive breast carcinoma was 23 per cent

2 The multicentric appearance of lobular carcinoma in situ concurrent with invasive breast carcinoma was 59 per cent

3 In 13 out of 16 cases lobular carcinoma in situ was found, also in the upper quadrants

4 Lobular carcinoma in situ occurred most frequently in highly differentiated carcino-

mas, but might occur in carcinomas of all degrees of differentiation

5 There was no correlation between size of tumour and appearance of lobular carcinoma in situ

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The author is grateful to Professor *Hemming Poulsen* M D, Chief Pathologist, for kindly permitting the use of breast tissue specimens from the Institute of Pathology, Copenhagen Municipal Hospital

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MULTICENTRIC AND BILATERAL APPEARANCE OF LOBULAR CARCINOMA IN SITU OF THE BREAST

A Retrospective Study of All Breast Tissue Specimens Removed from 52 Females with Lobular Carcinoma in situ

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During a follow up period averaging 17 years (2–30 years), 64 breast tissue specimens were removed in 52 females with lobular carcinoma in situ of the breast (LCIS) before, simultaneous with or subsequent to the detection of LCIS In 8 out of 25 cases the LCIS was bilateral, and in 9 out of 19 it was multicentric The present study does not reveal the actual multicentric and bilateral appearance of LCIS, but the "spontaneous" appearance in a series of specimens which were not removed or investigated primarily with the object of detecting this breast disease

Invasive breast carcinoma is often multicentric (*Qualheim* & *Gall* 1957, *Gallager* & *Martin* 1969), and the frequency of bilateral appearance ranges from 6 per cent to 10 per cent (*Robbins* & *Berg* 1964, *Leis et al* 1965, *Lewison* & *Neto* 1971)

As early as in their first report on lobular carcinoma in situ (LCIS), *Foote* & *Stewart* (1941) pointed out that also this breast disease was multicentric, and in 1959 *Barnes* reported the first cases of bilateral appearance

Newman (1963) found residual foci of LCIS in 28 out of 32 (86 per cent) mastectomy specimens and bilateral appearance in 6 out of 26 cases (23 per cent), in 3 of these cases simultaneously

Antonius & *Jones* (1963) demonstrated multicentric lesions in 28 cases (83 per cent) and bilaterality in 3 (9 per cent) Most of the 33 cases were LCIS and/or invasive lobular carcinoma

Among 13 cases, *Benfield et al* (1965) found residual foci of LCIS in 8 out of 10 mastectomy specimens (80 per cent) and bilateral appearance in 2 out of 5 examined cases

McDivitt et al (1968) suggested the multicentricity to be about 70 per cent, and simultaneous bilateral appearance 30 per cent

Urban (1967) proved that in 9 out of 26 cases examined (35 per cent), LCIS was present at random biopsy performed concurrently with mastectomy because of LCIS of the other breast

In 40 cases of LCIS, *Lewison* & *Finney* (1968) detected residual foci in 21 out of 34 mastectomy specimens (62 per cent) In 7 out of 15 cases examined they found, furthermore, bilateral foci of LCIS (46 per cent)

In 18 out of 61 cases *Hutter et al* (1969)

Received 26 xi 74 Accepted 26 xi 74

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found bilateral appearance of LCIS (29 per cent), and during the same year *Warner* (1969) made a survey of LCIS and found multicentric appearance to be about 70 per cent and bilateral not less than 15 per cent

Among 227 cases of LCIS, *Farrow* (1970) found residual foci in 63 per cent of mastectomy specimens LCIS was detected in the vicinity of the site of the biopsy, but also in other quadrants The bilaterality was 52 per cent (49 out of 94 cases examined)

Kaufmann et al (1971) detected residual foci of LCIS in 8 out of 12 mastectomy specimens and bilateral appearance in 2 out of 15 cases

In a series of LCIS with or without simultaneous invasive breast carcinoma, *Donegan & Perez-Mesa* (1972) studied the contralateral breast by means of elective biopsy in 23 cases and found 6 cases of LCIS (26 per cent)

Haagensen et al (1972) found 6 cases (25 per cent) with bilateral LCIS among 24 cases studied

In a series comprising 70 cases of invasive breast carcinoma *Andersen* (1974) found the multicentric frequency of LCIS to be 56 per cent

In this report both multicentric and bilateral appearance of LCIS will be assessed retrospectively on the basis of a series of breast tissue specimens which, contrary to most of the cases reported by the above authors, were all removed and examined primarily without regard to the presence of LCIS

MATERIAL AND METHODS

In a series of cases dating from the period 1 January 1942 to 1 October 1961 the diagnosis LCIS without simultaneous invasive breast carcinoma was established retrospectively in 52 females, as previously described (*Andersen* 1974) The present material originates from these 52 cases and represents breast tissue specimens removed earlier than at the same time as, or subsequent to the above mentioned breast tissue specimens The follow up period averaged 17 years (2 30 years) In only 3 cases was it impossible to trace the breast tissue specimens described in the original or in subsequent clinical records, in data obtained from the Danish Cancer Registry or in the files of the local hospital concerned The material is presumed to be complete, although in a few cases biopsy because of benign breast disease might have been done in hospitals outside the place of residence of the patient

Microscopical slides stained with haematoxylin eosin were prepared from all tissue blocks, and these were evaluated, as shown in Table 1 The criteria for LCIS and invasive lobular carcinoma have been described previously (*Andersen* 1974) Multicentric appearance of LCIS means LCIS detected in the same breast, but in breast tissue specimens removed at various times If mastectomy had been performed concurrently with the biopsy, the specimen is counted as one breast tissue specimen Consequently, the series comprises residual foci of LCIS in mastectomy specimens and foci in biopsies, which could be from the same lobule or quadrant as the original LCIS

RESULTS

The findings are presented in Table 1

The total number of breast tissue specimens was 64, 12 of which were percutaneous needle biopsies, 32 surgical biopsies, and 20 mastectomy specimens

Breast tissue specimens from the contralateral breast were available in 25 cases, 8 of which (32 per cent) presented LCIS, and in 19 cases breast tissue specimens were available from the ipsilateral breast, 9 of which (47 per cent) presented LCIS

LCIS was detected in a total of 20 breast tissue specimens 1 in percutaneous needle biopsies, 6 in surgical biopsies, and the remaining 13 in mastectomy specimens Hence, LCIS was demonstrated in 65 per cent of the mastectomy specimens, whether ipsilateral (7 out of 12 mastectomy specimens) or contralateral (6 out of 8 mastectomy specimens) In 7 breast tissue specimens, LCIS was detected without concurrent invasive carcinoma.

LCIS was demonstrated previously in 8, simultaneously in 1 (1 out of 5 examined cases), and subsequently in 11 breast tissue specimens the time being assessed on the basis of the breast tissue specimen included in the original series of LCIS

In addition, in the 64 breast tissue specimens fibroadenomatosis was found in 42,

TABLE 1 *Histological Findings in All Breast Tissue Specimens Removed from 52 Females with Lobular Carcinoma in situ of the Breast*

No	Year/age at original diagnosis of LCIS	Year when other breast tissue specimen were taken	Type of breast tissue specimen and number of tissue blocks	Ipsilateral/contralateral breast	Histological finding
3	1946/25	1955	NB/1	C	Unsuitable
–	–	1967	MS/1	I	LCIS, ILC, IDC, FIAD
5	1947/42	1942	B/1	C	FA
7	1948/36	1942	B/3	C	FIAD, DE
8	1949/45	1966	MS/5	I	LCIS, ILC
–	–	1973	B/1	C	ILC
9	1950/45	1955	NB/1	C	FIAD
11	1951/65	1951	NB/1	I	IDC
–	–	1951	MS/2	I	IDC
12	1951/40	1951	B/2	C	FIAD
13	1952/45	1965	B/1	I	IDC
	–	1965	MS/5	I	LCIS, IDC, FIAD
16	1953/43	1949	B/2	C	FIAD
18	1953/42	1953	B/2	C	FIAD
20	1954/50	1954	B/1	I	FIAD
	–	1954	B/2	C	FIAD
–	–	1969	B/2	C	LCIS, FIAD
21	1954/40	1953	NB/1	C	Unsuitable
–	–	1954	NB/1	C	Unsuitable
–	–	1954	B/2	C	FIAD
–	–	1965	B/5	I	FIAD
22	1954/42	1951	B/2	I	FIAD
–	–	1973	B/2	C	FIAD
23	1955/37	1954	NB/1	C	IDC
–	–	1954	MS/2	C	LCIS, FIAD
24	1955/42	1948	NB/1	C	
	–	1953	B/1	I	LCIS FIAD
–		1961	MS/4	I	LCIS, ILC, FIAD
26	1955/48	1948	B/2	C	FIAD
	–	1973	MS/2	I	IDC
27	1956/49	1955	NB/1	I	FIAD
31	1957/36	1961	MS/7	I	IDC
	–	1964	MS/4	C	IDC, FIAD
32	1957/55	1956	MS/3	C	LCIS, ILC
–		1957	B/1	I	LCIS, ILC, FIAD
–		1957	MS/2	I	LCIS, ILC, FIAD
33	1957/47	1957	B/2	C	FIAD
34	1958/39	1958	B/2	I	IDC, FIAD
–		1958	MS/1	I	LCIS, IDC, FIAD
35	1957/50	1953	NB/1	C	IDC
–		1953	MS/2	C	LCIS, IDC, FIAD
–		1953	NB/1	I	Unsuitable
–		1953	B/1	I	FIAD
–		1954	NB/1	I	Unsuitable
–		1955	NB/1	I	LCIS
36	1957/41	1963	MS/5	C	LCIS, DCIS, ILC, FIAD DE
37	1958/50	1952	B/1	I	FIAD
–	–	1952	MS/3	C	LCIS, DCIS, ILC
–	–	1953	B/1	I	LCIS, FIAD
–		1963	B/1	I	DE
38	1958/47	1958	B/1	C	LCIS FIAD

TABLE 1 (continued)

No	Year/age at original diagnosis of LCIS	Year when other breast tissue specimen were taken	Type of breast tissue specimen and number of tissue blocks	Ipsilateral/contralateral breast	Histological finding
39	1958/46	1960	B/4	I	LCIS, ILC, FA
–	–	1960	MS/2	I	LCIS, FIAD, FA
40	1958/41	1954	B/2	C	FIAD
–	–	1967	MS/2	C	IDC
41	1958/47	1956	B/1	I	FIAD, PAP, DE
–	–	1958	MS/2	I	FIAD, PAP
42	1959/74	1958	MS/3	C	LCIS, ILC, FIAD
44	1959–48	1952	B/1	C	FIAD
	–	1956	B/1	C	FIAD
–	–	1960	B/1	I	FIAD
–	–	1961	B/2	C	FIAD
47	1960/54	1960	MS/2	I	FIAD
–	–	1960	B/1	C	FIAD

NB needle biopsy
B surgical biopsy
MS mastectomy specimen
I ipsilateral
C contralateral

FIAD fibroadenomatosis
FA fibroadenoma
DE ductectasia
PAP papilloma
LCIS lobular carcinoma in situ
DCIS ductal carcinoma in situ
ILC invasive lobular carcinoma
IDC invasive ductal carcinoma

fibroadenoma in 3, papilloma in 2, ductectasia in 4, and invasive carcinoma in 24 (19 breasts) In 5 cases the tissue specimen available was too limited to allow of proper evaluation All cases except 3 presented fibroadenomatosis

DISCUSSION

The multicentric appearance of LCIS in the present series is thus somewhat lower than that stated in previous reports Presumably this is due to the fact that the number of tissue blocks available for this assessment is quite inadequate i e on an average less than 2 per breast tissue specimen In order to demonstrate this disease which is not recognizable by gross examination at least 10 tissue sections must be examined (*Lambird & Shelley* 1969, *Giordano & Klopp* 1973) Furthermore, during the studies previously reported the primary aim—and perhaps the predominant purpose—was to demonstrate LCIS in mastectomy species whereas this has not been the case during the primary investigation and the macroscopical selection of tissue sections for the present study

On the other hand, the bilateral appearance of LCIS is in complete agreement with previously reported studies, in spite of the fact that the above conditions also exerted an influence in these cases A likely explanation might be that the majority of cases of bilateral LCIS appear in patients with simultaneous invasive breast carcinoma—a feature which varies somewhat in the series previously mentioned However, if the appearance of LCIS in the entire series is compared this feature will hardly create any difference in multicentric and bilateral appearance in the various series On the other hand, if only part of the series is investigated, as in the present study the results might be influenced

Consequently, the present study does not

TABLE 1 *Histological Findings in All Breast Tissue Specimens Removed from 52 Females with Lobu Carcinoma in situ of the Breast*

No	Year/age at original diagnosis of LCIS	Year when other breast tissue specimen were taken	Type of breast tissue specimen and number of tissue blocks	Ipsilateral/ contralateral breast	Histological finding
3	1946/25	1955	NB/1	C	Unsuitable
–	–	1967	MS/1	I	I CIS, ILC, IDC, FIAD
5	1947/42	1942	B/1	C	FA
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11	1951/65	1951	NB/1	I	IDC
–	–	*1951*	*MS/2*	*I*	*IDC*
12	1951/40	1951	B/2	C	FIAD
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–		1957	MS/2	I	LCIS, ILC, FIAD
33	1957/47	1957	B/2	C	FIAD
34	1958/39	1958	B/2	I	IDC, FIAD
–		1958	MS/1	I	LCIS, IDC, FIAD
35	*1957/50*	*1953*	*NB/1*	*C*	*IDC*
–	–	1953	MS/2	C	LCIS, IDC, FIAD
–	–	1953	NB/1	I	Unsuitable
–	–	1953	B/1	I	FIAD
–	–	1954	NB/1	I	Unsuitable
–	–	1955	NB/1	I	LCIS
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–	–	1952	MS/3	C	LCIS, DCIS, ILC
–		1953	B/1	I	LCIS, FIAD
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	–	1958	MS/2	I	FIAD PAP
42	1959/74	1958	MS/3	C	LCIS ILC, FIAD
44	1959–48	1952	B/1	C	FIAD
–	–	1956	B/1	C	FIAD
–	–	1960	B/1	I	FIAD
–	–	1961	B/2	C	FIAD
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Consequently, the pre

reveal the true appearance of LCIS, but rather the "spontaneous" appearance in a series examined retrospectively

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LOBULAR CARCINOMA IN SITU

A Histological Study of 52 Cases

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In 52 cases of lobular carcinoma in situ of the breast (LCIS) without simultaneous invasive carcinoma, the actual lesion and the adjacent breast tissue were evaluated histologically and the findings related to the overall occurrence of invasive breast carcinoma No single histological parameter appeared to any significant degree in cases with invasive carcinoma, whereas, in a few instances, this was the case when certain parameters were found con currently However, with our present knowledge, the result of a histological subclassification of LCIS cannot be decisive in the choice of treatment Out of the 52 cases, 49 had fibroadenomatosis concurrently, of these, 6 were of a high degree of severity, 4 had fibro adenoma, 1 had papilloma, 8 had ductectasia, 5 non specific mastitis and 2 fat necrosis Only occasionally was intracellular mucin found in cases of LCIS, whereas PAS positive material was frequent In a few cases very large mast cells were seen in the adjacent stroma, but the significance of this finding remains unclarified

Since *Foote* & *Stewart* (1941) gave an excellent description of lobular carcinoma in situ of the breast (LCIS), only a few reports have been published on attempts to assess the histological variations of this disease, including the possible influence which the histological parameters may have on the prognosis Hence, *McDivitt et al* (1967) were "unable to correlate distribution of lobular carcinoma in situ within the excised specimen and the propensity of later infiltrative carcinoma" On the basis of the deviation of the cells from those found in normal glands, *Haagensen et al* (1972) classified LCIS into two types, one with smaller cells and of a uniform appearance (type A) and another with larger cells and poly morphism (type B) They regarded type A as requiring treatment to a lesser degree than type B, but stated now "This distinction has not, however, been founded on proof, and is of questionable value"

A few investigations concerning the ultrastructure are available (*Carter et al* 1969, *Schafer* & *Bassler* 1969, *Murad* 1971, *Ozello* 1971, *Tobon* & *Price* 1972), whereas no systematic studies into the common light-microscopical parameters in LCIS have ever been reported

Therefore, the present paper contains the results of such a histological assessment and attempts to correlate the findings and the overall occurrence of invasive breast carcinoma

Received 29 vi 74 Accepted 29 vi 74

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MATERIAL AND METHODS

The material consisted of 52 cases of LCIS with out simultaneous invasive carcinoma as described earlier (*Andersen* 1974)

The slides prepared for the present study were stained by the following methods haematoxylin-

eosin van Gieson, Alcian blue (*Eskelund* 1957) at pH 1 and 3, PAS with and without diastatic pre treatment, and toluidine blue

When relative sizes are given, comparison has in each case been made with the normal glands and epithelium of the adjacent glandular tissue

The criteria applied for the various benign breast disorders and the parameters included in fibroadenomatosis (cystic disease) are those de scribed in Relations of fibroadenomatosis to can cer of the breast (*Kiær* 1954) and Diseases of the breast' (*Haagensen* 1971)

In cases of invasive breast carcinoma it was not taken into consideration whether this disease had developed before or after the establishment of the diagnosis of LCIS in the breast tissue specimen concerned nor whether it was ipsilateral, contra lateral or both Only the overall occurrence of invasive breast carcinoma has been evaluated

In the statistical calculations the significance test for hypergeometric distributions was applied

RESULTS

The number of lobules involved in each individual case is shown in Table 1 In some cases the affected lobules were located in one area, but most often they were separated from each other In only 4 cases were the affected lobules so numerous and at the same time localized, that they must be presumed to have formed a tumour by themselves

TABLE 1 *Number of Affected Lobules in 52 Cases of Lobular Carcinoma in situ*

Number of lobules with lobular carcinoma in situ	Number of cases	Overall occurrence of cases with invasive breast carcinoma
1	4	1
2 4	18	5
5 10	14	2
>10	16	7

In 36 cases either individual involved lobu les or all of them were enlarged In 41 they were of irregular shape but in 11 cases also oblong In these 11 cases the lobules were often situated at the edge of a cyst and/or a fibroadenoma Within each individual lobule the ductules were uniformly large In 33 cases they were in all areas distinctly separated from the adjacent ductules, whereas in th remaining 19 cases some areas presented v sparse or no intermediary stroma at all

In 16 cases a few original lobular ductul with normal epithelium were d otherwise all ductules were affected in lobule in cases of LCIS

In all cases where it was possible to di ferentiate an intralobular duct, this was in volved in LCIS

In 41 cases there were also proliferat epithelial cells in lactiferous ducts of same appearance as those filling the lobul ductules (*Andersen* 1974)

In 46 cases the epithelial proliferation o the lobular ductules was solid and in 6 there was a *tendency to cribriform growth*

In 44 the proliferating epithelial cells w only slightly enlarged and the nuclei were uniform round and normochromatic, com pared to the size and appearance of the nor mal epithelial cells (type A) (Fig 1) In 8 the proliferating cells and their nuclei were on the other hand somewhat larger and

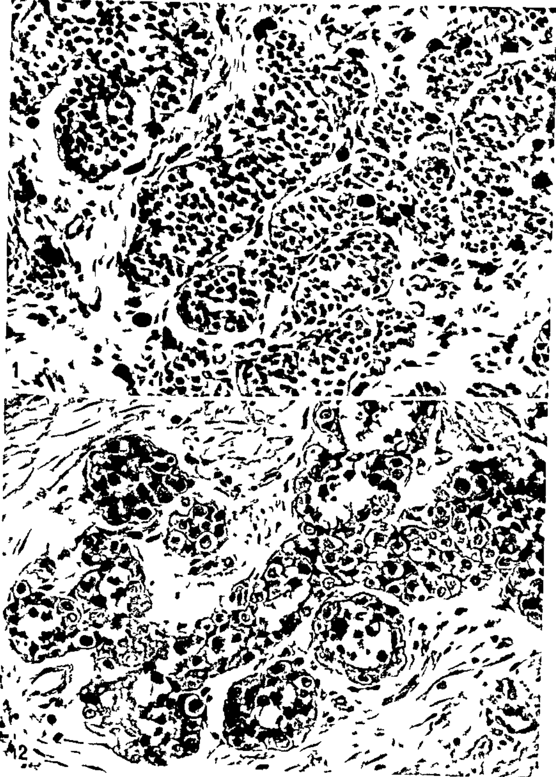
In 5, however, they could not be demon strated, in 2 of these presumably because technical errors had obliterated the nuclear structure In only 6 cases a few mitotic fig ures were found

In 11 cases the cytoplasm was marked eosinophilic whereas in the remaining 41 it was pale and vacuolized

In 43 cases the cellular cohesion was re duced to varying degrees and in 19 of these cases pycnotic epithelial cells were also found In 6 there was even small necroses in the

Fig 1 Lobular carcinoma in situ with solid pro liferation of uniform cells with round and normo chromatic nuclei (type A) Note the many large mast cells in the loose connective tissue Toluid ne blue 456 ×

Fig 2 Lobular carcinoma in situ with proliferation of cells with large somewhat polymorphic and hyperchromatic nuclei (type B) Haemat ylin eosin 456 ×


1
2

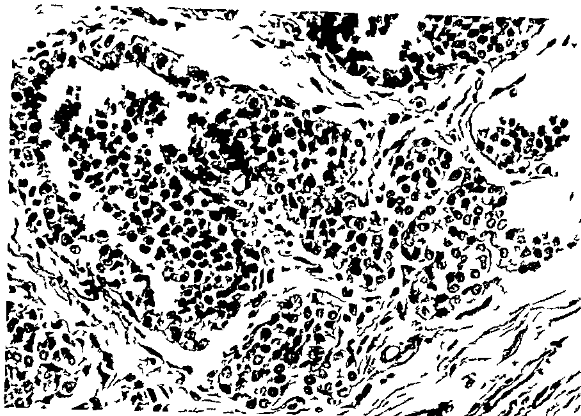


Fig 3 Lobular carc noma n s tu w th central necros s of the ep thel um Haematoxyl n-eos n 456 ×

central part of the proliferating ductular ep ithelium (Fig 3)

In all 52 cases flattened myoepithelial cells were found lying basal to the proliferating epithelial cells but within the so called base ment membrane Studies of the basement membrane in LCIS will form part of a forthcoming report

In 44 cases the lobular stroma was loose whereas in the remaining 8 cases it was firm ly fibrous

Microcalculi were demonstrated in 19 cases (36 per cent) In 10 less than 5 microcalculi were found whereas in the remaining cases there were more than 5 and most often a great many microcalculi They were situated most frequently in the adjacent glandular lumens or cysts but were also seen in the stroma In only 3 cases were they inside the LCIS

During significance tests Table 2) no histological parameters occurred singly to a significant degree in cases presenting overall occurrence of invasive breast carcinoma ($P \geqq 0\ 20$) If various histological parameters are combined 9 cases presented concurrently more than 10 lobules with LCIS enlargement of one or more of these and pycnot c cells and/or necrosis of the epithelium S x of these 9 cases had previously or subsequently developed invasive breast carcinoma whereas this was found in only 9 of the remain ng 43 cases ($0\ 05 > P > 0\ 02$)

In 4 of the 9 cases with more than 10 lobu les with LCIS there were concurrently lob i les with some nuclear polymorph sm and hyperchromatism (type B) All 4 h d previ ously or subsequently developed nvasive breast carcinoma whereas this was found in only 11 of the remaining 48 cases ($0\ 02 > P > 0\ 01$)

Hence the typical h stological p ture in LCIS was enlarged irregular lobules with evenly d stributed equally large ductules

TABLE 2 *Histological Findings in 52 Females with Lobular Carcinoma in situ and the Connection with Invasive Breast Carcinoma*

Histological parameters of lobular carcinoma in situ	Number of cases n = 52	Number of cases* with invasive carcinoma n = 15	Number of cases with subsequent invasive carcinoma		Number of cases with previous contralateral invasive carcinoma n = 5
			Ipsilateral n = 9	Contralateral n = 4	
Lobules					
Enlarged	36	13	7	3	5
Irregular shape	48	14	8	4	5
Oblonge shape	11	3	2	1	0
Ductules					
Closely packed	19	4	2	2	1
Remnants	16	5	4	2	0
Involvement of lactiferous duct	41	12	7	4	3
Type of epithelial proliferation					
Solid	46	14	8	3	5
Cribriform	6	1	1	1	0
Type A	44	11	6	4	4
Type B	8	4	3	0	1
Mitotic figures	6	2	1	0	1
Cytoplasm					
Pale and vacuolized	41	12	7	2	5
Eosinophilic	11	3	2	2	0
Reduced cell cohesion	43	13	8	4	4
Pycnotic cells	19	8	5	3	2
Necrosis	6	3	2	1	1
Lobular stroma					
Loose	44	12	8	4	3
Fibrous	8	3	1	0	2
Intraepithelial lymphocytes	31	11	6	3	3
Affection of more than 10 lobules enlargement of lobule and pycnotic cells	9	6	3	1	2
Affection of more than 10 lobules and Type B	4	4	3	0	1

* There was 3 cases with bilateral invasive carcinoma

filled with uniform, polygonal, large epithelial cells without stratification The nuclei were uniform, round, and slightly enlarged, but as a rule not particularly hyperchromatic The nucleoles were distinct and mitoses appeared infrequently The cytoplasm was pale and vacuolized, and the cellular cohesion was poor Most often the proliferating cells penetrated out into the terminal lactiferous duct In the peripheral part of the ductules flattened widely spaced myoepithelial cells were found lying inside the so called basement membrane The intralobular connective tissue was loose

The occurrence of benign breast disease appears from Table 3 In 3 cases of fibroadenoma LCIS was found within the lesion but this was also found concurrently in 2 cases in the adjacent glandular tissue In all the 5 cases with non-specific mastitis LCIS was found also in areas with slight or no inflammation

The occurrence of epithelial fibroadenomatosis parameters is shown in Table 4 Twenty had proliferation of the ductal epithelium and 5 of them had previously or subsequently developed invasive carcinoma, whereas 10 of the remaining 32 cases had

previously- or subsequently-developed invasive carcinoma (P > 0 20) Cases with fibroadenomatosis were classified according to severity from I to III (*Kiær* 1954) Grade III comprised only cases with pronounced intraductal epithelial proliferation, not borderline cases of intraductal carcinoma Forty three cases were of grades I–II severity, whereas 6 were of grade III One of these developed invasive breast carcinoma

TABLE 3 *Benign Breast Diseases in 52 Cases of Lobular Carcinoma in situ*

	Number of cases
Fibroadenomatosis	49
Fibroadenoma	4
Papilloma	1
Ductectasia	8
Non specific mastitis	5
Fat necrosis	2

TABLE 4 *Epithelial Fibroadenomatosis Parameters in 52 Cases of Lobular Carcinoma in situ*

	Number of cases
Macrocysts	22
Microcysts	40
Apocrine metaplasia	41
Localized adenosis	37
Blunt duct adenosis	36
Papillomatosis	28
Proliferation of duct epithelium	20
Tendency to fibroadenoma	6
None of the above parameters	3

In 31 cases we succeeded producing Alcian blue stained slides of LCIS In only 3 cases was there a faint intracytoplasmic reaction both at pH 1 and 3 In the same 31 cases we succeeded also in producing a PAS reaction This was negative in 5 cases corresponding to the proliferating epithelium in LCIS On the other hand it was positive in 26 cases corresponding to the intracytoplasmic coarse granules and there was also a faint accentuation corresponding to the [illegible]ular border in the proliferating epithelium [illegible] strength of the PAS reaction tended to abate when th[illegible] tissue had been pre treated with diastase

In the specimens stained with toluid[illegible] blue, very large mast cells filled with gran[illegible] were seen in the loose intralobular connecti[illegible] tissue in some cases with LCIS (Fig 1), similar cells could also be seen in the co[illegible]nective tissue of the adjacent lobules and the loose periductal connective tissue [illegible] cause the slides were not produced with th[illegible] object of semi quantitative evaluation of [illegible] mast cells and because the composition o[illegible] the previously applied fixation methods w[illegible] not known for certain, I abstained from ar[illegible] actual quantitative analysis of the mast cell and a study of the metachromatism of the stroma However, in 4 cases there were many mast cells both in relation to lobules with lobular carcinoma in situ and to other lobules Three of these cases developed invasive carcinoma

DISCUSSION

This investigation showed that no decisive prognostic importance could be attached to any individual histological parameter Only in a few of the 52 cases (17 per cent) did the concurrent appearance of several histological parameters seem to be significantly related to the overall occurrence of invasive breast carcinoma, but in by far the majority of cases with LCIS this did not apply

There were several reasons for applying the overall occurrence of invasive carcinoma in the assessment of any possible significant relationship between the histological parameters and invasive breast carcinoma, whether previous or subsequent, or ipsilateral or contralateral 1) Invasive breast carcinoma has a pronounced tendency to multicentric and bilateral occurrence (*Qualheim* & *Gall* 1957, *Robbins* & *Berg* 1964, *Gallager* & *Martin* 1969, *Urban* 1969 *Hutter* & *Kim* 1971) This means that breast carcinoma is not a focal process but a diffuse disease of the entire breast epithelium 2) Invasive breast carcinoma has presumably most often a very prolonged preclinical course (*Collins*

et al 1956, *Schwartz* 1961) This means that the date of the actual detection of LCIS and any clinical demonstration of invasive breast carcinoma seems to be of lesser importance By way of example it should be stated that if invasive carcinoma is detected one year before LCIS and/or one year after, it is highly probable that the invasive carcinomas and the LCIS were present concurrently in the breasts

The high frequency of fibroadenomatosis in the present series is in agreement with the fact that the majority of patients appeared primarily with complaints caused by this disease On the other hand, lobular carcinoma in situ was demonstrated incidentally at histological investigation Therefore, concurrent appearance of lobular carcinoma in situ and fibroadenomatosis does not necessarily indicate a common genesis

A histological sub classification of LCIS seems therefore, to be of limited value only, and based on our present knowledge, this procedure cannot be decisive for the choice of therapeutic measure

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The author is grateful to *Ole Horwitz*, M D, Director of the Danish Institute of Clinical Epidemiology for helpful advice and valuable statistical assistance

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Acta path microbiol scand Sect A, 82 742 746, 1974

CONCENTRATIONS OF RENIN AND RENIN SUBSTRATE IN PLASMA OF RABBITS DURING PREGNANCY AND THE POST PARTUM PERIOD

The Mechanism of the Rapid Pronounced Decrease in Uterine Renin Content post partum in Rabbits I

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The content of renin in rabbit uterus is known to increase many fold during pregnancy followed by a rapid fall to normal levels within a few days after delivery In order to study this mechanism the blood concentrations of renin and its substrate were measured Contrary to the concentration of renin in uterus, the plasma renin concentrations were not increased either during pregnancy or in the post partum period The renin substrate concentration was unchanged during the first part of pregnancy but was increased 2 3 fold in the days around delivery This increase resulted in a corresponding increase in plasma renin activity It was concluded that the rapid inactivation of uterine renin post partum was due to a local inactivation and not to a release of renin to the blood

High concentrations of renin have been found in the uterus of several mammals (*Stakemann* 1960 and *Gross et al* 1964) and in the human uterus (*Skinner et al* 1968) In the rabbit the uterine renin concentration falls from very high levels to non pregnant levels during the first two days post partum (*Bing & Faarup* 1966)

The aim of this and the following study has been to investigate if this rapid and marked fall after delivery can be explained by a release of renin to the blood or whether local factors are involved in the inactivation of uterine renin

For this purpose the concentration of renin in rabbit plasma was followed in the non pregnant state, during pregnancy and in the post partum period In the same animals renin substrate concentration and renin activity were determined

MATERIAL AND METHODS

Animals 9 pregnant and 17 non pregnant albino country rabbits (The State Serum Institute) were used

Peripheral blood the blood (1½–2 ml to which was added 50 µl 0 17 M sodium citrate per ml of blood) was withdrawn from the ear artery of the conscious rabbits using a hypodermic needle In a few cases blood was obtained by cutting the ear artery or the main ear vein Immediately after sampling the blood was cooled to 0° C and centrifuged

The plasma samples were stored at –20° C

Renin assay was based upon the principle of de

Received 27 vi 74 Acc[illegible]ted 7 [illegible] 74

Requests for repr[illegible]ts [illegible] be addressed to *Jorgen Jorgensen*, The [illegible]ty Institute for Experimental Medicine [illegible] 71 DK 2100 Copenhagen Ø, Denmar[illegible]

termination of the decrease in renin substrate in the course of time using radioimmunoassay for the estimation of angiotensin I (*Poulsen* 1969 a, b) as slightly modified by *Eskildsen* (1972). Renin concentration was expressed in GU $\times 10^{-4}$ per ml plasma by reference (*Eskildsen* 1973 a) to a purified hog renin standard preparation kindly supplied by Dr Haas and identical with that donated by Dr Haas to the National Institute for Biological Standards and Control, Holly Hill, Hampstead, London.

The velocity of the reaction between substrate and renin in the rabbit plasma has been found to obey first orders kinetics with respect to substrate concentrations to at least 1200 ng angiotensin per ml of plasma (equal to 1.2×10^{-6} M) (*Ryan & McKenzie* 1968). This corresponds to the Km values for uterine and kidney renin which have been found to be 1.5×10^{-6} and 5×10^{-6} M respectively (*Anderson et al* 1968). Thus, the renin concentrations were calculated according to the integrated standard I order equation.

Renin substrate concentration was expressed as nanograms angiotensin I per ml plasma. *Renin activity* was calculated as renin concentration (k × E) times substrate concentration (So) and expressed in nanograms angiotensin I formed per ml of plasma per hour of incubation.

The day to day variation of the assay was estimated by repetitive assays of the same sample and showed (n = 24) a SD of ± 17 per cent for substrate and renin concentrations and a SD of ± 27 per cent for renin activity.

RESULTS

Non pregnant Rabbits

The renin parameters were followed in 17 normal non pregnant rabbits. The *renin concentration* was in mean $1.3 \pm 0.5 \times 10^{-4}$ GU $\times$ ml^{-1} (mean ± SD, n = 17), the range being $0.45–2.8 \times 10^{-4}$ GU $\times$ ml^{-1}. The *renin substrate concentration* was 340 ± 50 ng $\times$ ml^{-1} (mean ± SD, n = 17), range 190–550 ng $\times$ ml^{-1}. The *renin activity* was 5.6 ± 2.3 ng $\times$ ml^{-1} $\times$ h^{-1} (mean ± SD, n = 17), range 1.1–16.8 ng $\times$ ml^{-1} $\times$ h^{-1}.

In order to measure the day-to-day variations in these parameters through the experimental period, 3 rabbits were bled frequently during a 7 week period. The concentrations varied only moderately and showed no cyclic variation. The relative variations in the individual animals were for *renin concentration* ± 27, 39 and 41 per cent SD (n = 16, 14 and 15 respectively). The *substrate concentration* varied ± 17, 21 and 18 per cent SD (n = 16, 15 and 15) and the *renin activity* varied ± 42, 58 and 47 per cent SD (n = 16, 14 and 15).

Pregnant Rabbits

The same parameters were studied in 9 pregnant rabbits 5–29 days before delivery. It is seen from Fig. 1 that the *renin concentrations* were within the normal non pregnant range except for a few instances where higher values were measured. In the last 5 days of pregnancy, where the content of renin in uterus is known to be excessively high, the plasma *renin concentration* was in mean $1.4 \pm 1.2 \times 10^{-4}$ GU $\times$ ml^{-1} (n = 19). This value was not significantly different from normal non pregnant values ($p > 0.6$, Students' T test).

The *renin substrate concentration* was rather constant and within the non pregnant range during the first part of pregnancy. However, during the last 5 days of pregnancy the substrate concentration increased 2–3 fold, the mean concentration being 570 ± 230 ng $\times$ ml^{-1} (n = 19), significantly higher than the normal non pregnant level ($p < 0.01$).

The *renin activity* was at the start of pregnancy within the non pregnant range. During the last 5 days before delivery the mean renin activity increased to 10.6 ± 8.9 ng $\times$ ml^{-1} $\times$ h^{-1} (n = 19), significantly higher ($0.05 > p > 0.01$) than the non pregnant value.

Rabbits post partum

The 9 pregnant animals were followed with repetitive blood samples for periods of 1–21 days after delivery. The results for the first 11 days are shown in Fig. 1.

The *renin concentration* was not increased at any time. During the first two days post partum, where the marked fall in uterine renin content occurred, the mean value was $1.2 \pm 0.5 \times 10^{-4}$ GU $\times$ ml^{-1} (n = 18) which was not significantly different from

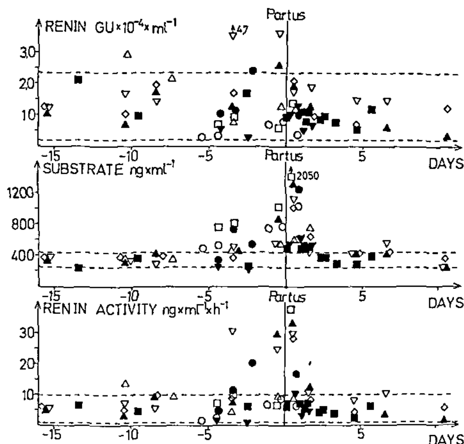


Fig 1 Renin and substrate concentration and renin activity in peripheral plasma of rabbits during the *[la]st half of pregnancy and the first 11 days post partum* Different symbols represent individual animals The dashed lines give the 95 per cent confidence limits of 17 non pregnant rabbits

the normal non-pregnant values ($p > 0.6$)

Substrate concentration was markedly elevated ($p < 0.01$) during the first 2 days post partum The mean concentration during this period was 800 ± 430 ng per ml (n = 18) After the 4th day following parturition values in the non-pregnant range were found

The *renin activity* was in the first two days 13.6 ± 11.0 ng × ml⁻¹ × h⁻¹ (n = 18), significantly higher ($p < 0.01$) than in the non-pregnant animals After this period values were in the non-pregnant range

DISCUSSION

The rapid fall of the renin concentration in the rabbit uterus during the first 2 days post partum (*Bing & Faarup* 196[?]) implies that with a uterine weight of 50 g and a renin concentration of at least 10 GU per g at partus, more than 450 GU renin disappears from the uterus within this period These large amounts of renin, several fold greater that the total content of the kidneys (50–100 GU) could be inactivated locally or escape to the blood in active form, through the veins or the lymphatic system The escape of such an amount of renin to the blood would, with a constant rate of release during the first two days post partum imply that at least $\frac{450}{48 \times 60} = 0.16$ *GU* would leave uterus every minute With a plasma volume of 180 ml, this corresponds to $\frac{0.16}{180} = 8.9 \times 10^{-4}$ GU per minute per millilitre of plasma The half-

life for renin in plasma has been found to be 9 min in the rat (*Peters-Haefeli* 1971) and 30 min in the dog (*Goldblatt et al* 1972) Calculated for a half life of 9 min, $\frac{\ln 2}{9} = 0.077$ will be the fraction of renin in plasma removed per min This will give equilibrium at a renin concentration in plasma of $\frac{8.9 \times 10^{-4}}{0.077}$ $= 115 \times 10^{-4}$ GU per millilitre in addition to renin secreted by the kidneys

In the samples from the early post partum period a mean plasma renin concentration of 12×10^{-4} GU per millilitre was found This value is less than one per cent of the above mentioned possible levels and is not significantly different from the non-pregnant values *indicating that the uterine renin is not* or only in minimal amounts secreted to the blood.

In the pregnant state rather high concentrations of renin were found in a few cases This could be explained as occasional release of renin from the uterus or kidneys to the blood High concentrations of renin in uterine venous blood of pregnant rabbits have been found by *Eskildsen* (1973 b) and after different stimuli by *Ferris et al* (1972) *Eskildsen*'s finding of slightly elevated renin concentrations in peripheral plasma on the 28th day of pregnancy could not be reproduced in the present investigation

The marked increase in *renin substrate* concentration found just before and after parturition has been demonstrated in late pregnancy by *Eskildsen* (1973 b) and is also known from the human pregnancy (*Helmer & Judson 1967, Weir et al 1970, Skinner et al* 1972) The elevated substrate concentration with unaltered renin concentration is the cause of the increased renin activity found in this stage of pregnancy

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This work was supported by grants from the *Danish State Medical Research Council* and the *Danish Heart Association* (to *Jorgen Jørgensen*) and from *King Christian X Foundation* (to *Jens Bing*)

The author is grateful to *Knud Poulsen* M D for valuable discussion and to Mrs *Jane Czuba* and Mrs *Dorthe Kolding* for valuable technical assistance

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Acta path microbiol scand Sect A, 82 747–759, 1974

EVALUATION OF METHODS FOR SPECIFIC LOADING OF KUPFFER CELL LYSOSOMES WITH HEAVY COLLOIDAL PARTICLES

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The purpose of this electron microscopic study was to analyze the possibility to achieve a selective uptake of heavy macromolecules in Kupffer cell lysosomes of rat liver in order to create a basis for attempts aiming at isolating Kupffer cell lysosomes by way of ultra centrifugational techniques Intravenous injections of colloidal solutions of thorium dioxide and silver iodide were employed for this purpose The results showed that typical thorium dioxide macromolecules (70–200 Å large) appeared in the vacuolar apparatus of both Kupffer and parenchymal cells early after the injection On the other hand, aggregates of silver iodide macromolecules (200–1,300 Å large) were only encountered in the Kupffer cells, and became concentrated in the lysosomes of these cells during the first hour after intravenous administration Disappearance of the large granular silver iodide compound occurred between 12 and 24 hours post injection The evidence suggested that this was due to disaggregation of the macromolecules into single silver iodide units capable of escaping through intact lysosomal membranes These small molecules might—in part—be transferred to the lysosomes of the parenchymal cells It was concluded that pretreatment of rats with an intravenous injection of colloidal silver iodide (Neosilvol®) should be a useful approach to achieving the isolation of Kupffer cell lysosomes, provided the fractionation was performed approximately 60 minutes after the administration

Comparatively pure fractions of lysosomes can be obtained from rat liver by homogenization following procedures which alter the density of the lysosomes Such procedures include pretreatment of experimental animals with Triton WR 1339 Dextran and iron sorbitol citric acid complex (Jectofer®) (2 4, 7, 22) With all these procedures it appears likely that the lysosomes both in Kupffer cells and parenchymal cells are isolated together in the same fraction

Hepatocytes (or parenchymal cells) and sinusoidal lining (or reticuloendothelial) cells represent the two major components of mammalian liver Thus, it has been calculated that hepatocytes constitute 90–95 per cent of hepatic cellular weight (but only 60–65 per cent of the total liver cell population), while sinusoidal lining (essentially Kupffer and "endothelial") cells constitute 5–10 per cent of liver weight and approxi-

Received 20 v 74 Accepted 10 vi 74

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mately 35 per cent of the total cellular population (14 19) It would obviously be of interest to be able to study the populations of lysosomes in the above two cell types separately, since they emanate from cells with widely differing functions

Kupffer cells and endothelial cells both are capable to ingest and store macromolecules which are present in the blood circulating through the sinusoids, although to a somewhat variable extent—and depending on the cell type (25) According to many investigators, the presumed endothelial cells can be regarded as precursors of the Kupffer cells under normal as well as pathological conditions (for a review, see ref number 5) However, Wisse (25) and others claim that Kupffer and endothelial cells represent two unrelated types of cell This controversy has so far not been resolved From the functional point of view, the Kupffer cells appear to differ from the endothelial cells only in their greater phagocytic avidity (8) At present, it therefore seems most practicable not to discriminate between the two cell types but merely regard them as two closely interrelated variants—especially in view of the obvious difficulties to identify them ultrastructurally (25) There is no indication that small endothelial like cells become more concentrated than the other in the "Kupffer cell" or nonparenchymal cell fraction which can be isolated from mammalian liver (1) Taking this into account, and for the sake of convenience and simplicity in presentation we have chosen to refer to these cells as Kupffer cells bearing in mind their possible difference in histogenesis

Although studies and comparisons of isolated parenchymal and Kupffer cells have suggested that differences in the enzymic composition of the lysosomal enzymes in these cells might occur (3 9) it has so far not been possible to isolate the lysosomes from *the two types of cells in reasonably pure form* The purpose of the present study was to analyze the possibility to achieve a selective uptake of macromolecules in Kupffer cell lysosomes as revealed by ultrastructur methods By so doing, a basis would be created f subsequent attempts to isolate Kupffer ce lysosomes by way of ultracentrifugati fractionation methods Preliminary (7, 11, 12) have indicated that two mac molecules would be particularly useful this respect colloidal thorium dioxide ar silver iodide macromolecules

MATERIAL AND METHODS

Male Sprague Dawley rats were used througho the study They were maintained on ordinary l ratory rat pellets until approximately 15 hour prior to sacrifice and were allowed water *ad lib*

Thorotrast® (Testagar, Detroit, Mich, U S A (a solution composed of 24 26 per cent colloida thorium dioxide stabilized with 25 per cent trin) diluted 1 1 with isotonic saline was inj into a mesenteric vein—to Nembutal® anesthetized, laparotomized animals—or intravenously (ir a sublingual vein) in an amount corresponding to 0 5 ml stock solution per 100 gm of body weight Mesenteric vein injections were only performed when the brief intervals (1, 2, 3, and 5 mins) were to be studied, while injections in the sublingual vein were used for the longer intervals (10, 20, and 30 mins, and 1, 6, 12 24, 48, and 72 hours)

Neosilvol® (Parke, Davis & Co, Detroit, Mich, U S A) (~30 per cent colloidal silver iodide stabilized with gelatin) was injected intravenously (in a mesenteric or sublingual vein during ether or Nembutal® anesthesia) in a dose of 12 5 mg per 100 g of body weight The intervals studied were 3, 6, 10, 20, and 30 mins, and 1 3 6 12, 24, 48, 72, and 168 hours

At the time of sacrifice, the livers were prepared in either of two ways (*a*) The rats were anesthetized with Nembutal®, the abdomens opened, and the livers perfused by fixation with 2 per cent purified glutaraldehyde in 0 15 M cacodylate buffer Following perfusion for about 1° min, thin pieces of adequately fixed liver tissue were excised and further fixed for 24 hours in the glutaraldehyde solution These tissues were either utilized for the demonstration of acid phosphatase (see below) or washed in the cacodylate buffer and post fixed in a 2 per cent *s* collidine buffered OsO_4 (0–4° C, pH 7 4) (*b*) Animals were killed by a blow on the head and small cubes of liver tissue *(with a size not exceeding 1 mm)* were directly immersed in 2 per cent OsO_4, as described above

For the histochemical demonstration of acid phosphatase, slices of glutaraldehyde perfused liver were treated with 10 per cent DMSO in buffer for 24 hours (17) Approximately 50 μ thick sec

tions were then prepared on a regular freezing microtome and were incubated in a Gomori type medium as described by Barka and Anderson (6) for periods ranging between 30 and 60 mins The sections were subsequently washed and postfixed in OsO_4 (see above) Controls were incubated in a medium lacking the substrate

Determination of silver in peripheral blood and liver At different time periods after the injection of Neosilvol® (5 mins to 7 days) the animals were anesthetized and samples of blood were collected The blood in the liver was then washed out by perfusion of the portal vein with isotonic saline and pieces of liver tissue were subsequently removed for silver determination The samples to be examined were placed in a porcelain crucible dried and charred by heating, at first at low temperature, then at 800° C 2 ml concentrated sulphuric acid and 0 5 ml nitric acid was added and the mixture was evaporated The addition of acids and the evaporation was repeated once, and a small volume of nitric acid and 10–15 ml of hydrochloric acid was added to the material in the crucible The content of silver in the solution was measured by atomic absorption at 328 nm Silver nitrate was used as standard

RESULTS

A *Silver Measurements*

The results showed that silver disappeared quickly from the blood stream after reaching a peak value immediately after the administration of Neosilvol® Thus, 1 hour after the injection, the concentration of silver was only about 15 per cent of that found at the 10 minute interval (not shown in figure) As illustrated in Figure 1—showing the concentration of silver in liver tissue at various intervals after Neosilvol® injection—the values recorded during the first hour were high A slight decrease in silver concentration was noted after 24 hours During the following 2–7 days, the amount of silver in the liver decreased steadily, and on day 7 only about 10 per cent of the concentration reached 30–60 mins after the injection was recorded

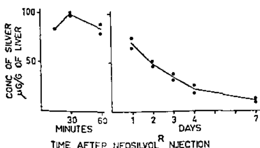


Fig 1 Diagram illustrating the concentration of silver in the liver at different intervals after the injection of Neosilvol® intravenously

B *Thorotrast®, Kupffer Cells*

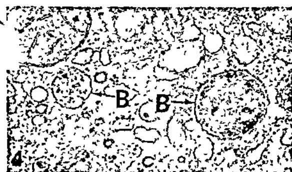
At the earliest interval studied—1 minute after mesenteric vein injection—typical electron dense thorium dioxide macromolecules with a diameter varying between approximately 70 and 200 Å were found to be aligned along portions of the surface of the Kupffer cells Similar macromolecules were present in small invaginations and "coated pits" formed by the plasma membrane Occasionally, small vesicles and ovoid or tubular elements located immediately below the plasma membrane were filled with Thorotrast® particles The appearance was approximately the same 2 (Fig 2) and 3 mins after the injection, although the number of intracellular vesicles and tubular structures containing particles was slightly increased in comparison with the situation at the 1 minute interval

Figure 3 illustrates the characteristic appearance 10 mins after an intravenous injection of Thorotrast® It will be seen that many of the thorium dioxide macromolecules still adhere to the surface of the cell, while the cytoplasm is filled with vesicles and vacuoles of variable size containing densely packed macromolecules In other cells, there were abundant tubular elements showing presence of Thorotrast® particles, in addition to the vesicles and vacuoles Although acid phosphatase might be present in some of the largest vacuoles, most vesicles and vacuoles seemed to lack the enzyme

At the 30 minute interval, most Kupffer cells showed presence of abundant particle-filled vesicles vacuoles, and bodies in their cytoplasm, as illustrated in Figure 7 Many of the largest of these structures had a homogeneously dense matrix and contained membranous, vesicular, and dense inclusions in

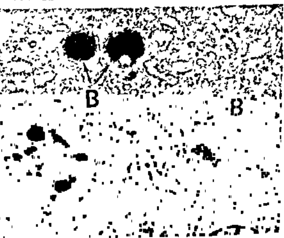

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addition to the thorium dioxide macromolecules Following incubation for the demonstration of acid phosphatase, most of the particle-containing vacuoles and bodies appeared to be covered with reaction product, which—however—was lacking in the small vesicles (diameter below approximately 0 2 μ)

The results were in general the same 1 hour after the injection, while at later intervals (up to 72 hours) the small vesicles containing electron dense macromolecules had disappeared Final product resulting from incubations in the Gomori-type medium usually covered most or all of the particle filled vacuoles and bodies

C *Thorotrast®, Parenchymal Cells*

No Thorotrast® particles were observed within the parenchymal cells until 30 mins after the injection At this interval, small numbers of particles were found in occasional lysosome-like bodies, usually located in the peribiliary region (Fig 4) Later after the injection, more abundant particles were observed in such bodies, and the number of particle containing bodies tended to increase with the time after the injection, so that at 24 hours most or all of the lysosome like bodies contained particles (Figs 5 and 6) Following incubations for the demonstration of acid phosphatase, most or all of these bodies showed presence of reaction product The appearance at 48 and 72 hours was inseparable from that at 24 hours

D *Neosilvol®, Kupffer Cells*

The binding to, and subsequent uptake and transport within, Kupffer cells of the electron dense 200-1,300 Å large silver iodide particles appeared to follow the same general pattern as concerning thorium dioxide macro molecules (Figs 8–11) Already 10 mins after the injection, some of the silver iodide-containing vacuoles showed presence of deposits of lead phosphate following incubation for the demonstration of acid phosphatase At

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Fig 2 Thorotrast® intraportally, 2 minutes Superficial portion of Kupffer cell with dense thorium dioxide macromolecules adhering to the surface and appearing intracellularly in small vesicular and tubular structures (*arrows*) OsO_4, Epon, section staining with lead citrate × 25,600

Fig 3 Thorotrast® intravenously, 10 minutes Picture illustrating a Kupffer cell with Thorotrast® particles adhering to the surface and occurring in intracellular vesicles and vacuoles of variable size OsO_4, Epon, section staining with lead citrate × 24,300

Fig 4 Thorotrast® intravenously 30 minutes Golgi region of a parenchymal cell with 2 bodies (B) (presumably lysosomes) containing electron dense particles of a size corresponding to that of thorium dioxide macromolecules OsO_4, Epon section staining with lead citrate ×27,000

Fig 5 Thorotrast®, 6 hours Lysosome like bodies (B) contain numerous granules presumed to represent thorium dioxide macromolecules OsO_4 Epon section staining with lead citrate × 21,500

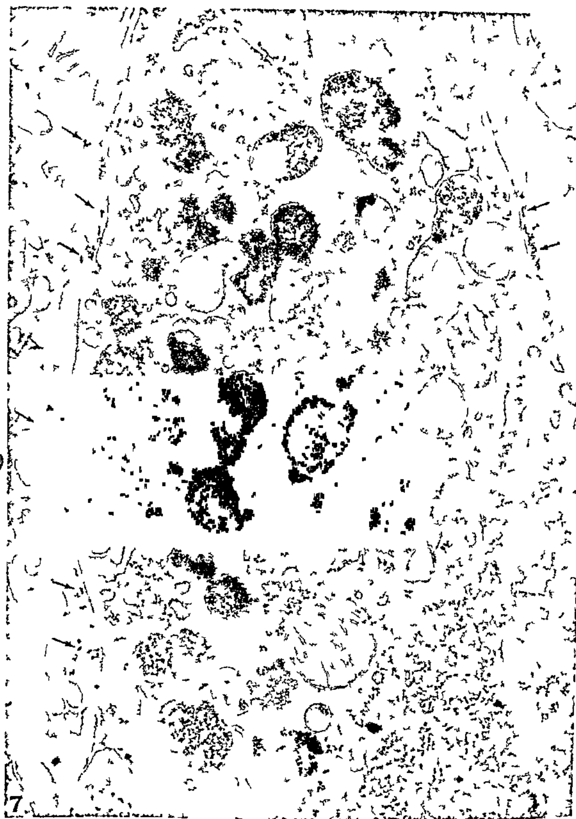
Fig 6 Thorotrast® intravenously, 24 hours Portion of parenchymal cell with several macromolecule-containing bodies (B) OsO_4, Epon section staining with lead citrate × 23 000

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Fig 7 Thorotrast® intravenously, 30 minutes Portion of Kupffer cell containing abundant variously sized Thorotrast® filled vesicles, vacuoles and bodies Some thorium dioxide macromolecules are also present extracellularly (*arrows*) OsO_4, Epon, section staining with lead citrate × 29,400

Fig 8 Neosilvol® intraportally, 3 minutes Portion of Kupffer cell with silver iodide particles adhering to the surface and appearing in tubular, vesicular, and vacuolar structures intracellularly OsO_4, Epon section staining with lead citrate × 23,000

Fig 9 Neosilvol® intravenously, 10 minutes glutaraldehyde fixed tissue incubated for the demonstration of acid phosphatase (incubation time 30 minutes) Reaction product appears as clusters of fine needles In the portion of the Kupffer cell illustrated, these needles are present in at least three of the bodies (B) which also contain granules with a structure indicating that they represent silver iodide particles Similar particles are also present in two vacuoles (V) which do not seem to show reaction product Perfusion fixation with glutaraldehyde, frozen sections incubated in the modified Gomori medium, postfixation in OsO_4 Epon, section staining with lead citrate × 31,500


7

B

V

V

B

B

9

later intervals, as long as silver iodide particles were still present within the cells (see below), most or all of the macromolecule-containing bodies appeared to carry acid phosphatase

Beginning 6 to 12 hours after the injection, a steadily decreasing number of macromolecule-filled bodies were observed in the Kupffer cells, so that at 48 to 72 hours typical granules were usually not present in the lysosome-like bodies in the Kupffer cells (Fig 13) In some rats, the appearance was the same already 24 hours after the injection However, many of the lysosome-like bodies showed diffusely increased opaqueness of their matrix which appeared to contain a finely granular material

E *Neosilvol®, Parenchymal Cells*

The large, electron dense particles observed in the Kupffer cells and believed to represent silver iodide macromolecules were never encountered within the parenchymal cells The fine structure of these cells was unaltered until (6) 12 hours after the injection, when of the lysosomes were found to contain finely granular material (approximative of individual granules 40 Å) This granular material within the lysosomes became more conspicuous at later intervals (Fig 12) and was still present 72 hours after the injection Otherwise, the fine structure of the parenchymal cells was unchanged

F *General*

The fine structural appearance of Kupffer and parenchymal cells and the localization of thorium dioxide and silver iodide macromolecules was the same irrespective of whether primary fixation was performed with glutaraldehyde or osmium tetroxide In the histochemical studies of the localization of acid phosphatase, deposits of final product were constantly lacking in the control sections (incubated without β glycerophosphate in the medium)

DISCUSSION

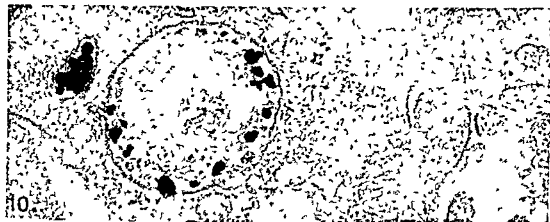
The findings in the present study indi that thorium dioxide and silver iodide m molecules are taken up by the Kupffer cell in the same manner and at approximately similar rates Thus, the particles first adh to the surface of the cells and subsequently become enclosed in vesicles, tubules a vacuoles through formation of invaginatic of the plasma membrane (11–13) The hi stochemical evidence suggested that most of these endocytotic vesicles and vacuoles as well as the tubules frequently and rapidly fused with lysosomes In this way most of the Kupffer cell lysosomes appeared to have become filled with macromolecules 30 minutes after the injection of Thorotrast® and Neosilvol®

A distinct difference was noted between silver iodide and thorium dioxide molecules with respect to their relations to the parenchymal cells while the 200–1,300 Å silver iodide particles never were encountered in the cytoplasm of these cells, typical thorium dioxide particles appeared in lysosome like elements 30 minutes after the injection and tended to increase in number up to the 12 and 24 hour intervals (21) The reason for this discrepancy between the two macromolecular substances may simply be their difference in size, the silver iodide molecules were too large to penetrate through the endothelial pores to reach the space of Disse (*cf* 24)

Silver iodide and thorium dioxide also differed with respect to the behaviour within the

Fig 10 Neosilvol® intraportally, 3 minutes Small area of Kupffer cell with two vacuoles containing silver iodide particles In addition to the dense particles, the larger of these vacuoles also contains some granular and membranous material with a structure often seen in lysosomes OsO_4, Epon, section staining with lead citrate × 85,000

Fig 11 Neosilvol® intravenously, 1 hour A Kupffer cell containing several large bodies and vacuoles containing electron dense silver iodide particles Note presence of dense and membranous materials in some of the bodies OsO_4, Epon, section staining with lead citrate × 23,000



11

12

V

13

Kupffer cells while the thorium dioxide particles were retained in the cells for as long period of time as the observations lasted (72 hours), the 200 1,300 Å silver iodide macromolecules regularly had disappeared already 24 hours after the injection, and were clearly reduced in number at the 12 hour interval The reason for this discrepancy is not readily apparent One explanation may be that the Kupffer cells rid themselves of silver iodide by way of exocytosis (reverse endocytosis) while they are unable to do the same with the thorium dioxide particles (10) However, it appears unlikely that the lysosomal system would react differently in the handling of these two inert substances only because of a small difference in size of the enclosed particles Furthermore, carbon particles—which are of a size corresponding to that of the silver iodide macromolecules—are retained in a similar fashion as thorium dioxide particles (15, 23)

Another explanation might be that the Kupffer cells containing silver iodide detach from the sinusoids, move away with the blood stream and are rapidly replaced by cells either emanating from other sinusoidal lining cells or by macrophage-like cells brought to the liver via the portal blood (18, 20) Alternatively, disappearance of Kupffer cells containing silver iodide might be caused by a toxic action of this substance resulting in necrosis and dissolution of the cells and their contents Although the present findings do not exclude disappearance of silver iodide-containing Kupffer cells from the liver during the 24-hour period after the injection (either due to detachment or dissolution and necrosis), this hypothesis appears unlikely for the following reasons (*a*) If the silver iodide were toxic and caused necrosis of the Kupffer cells, silver iodide macromolecules would be released from the disintegrating cells, recirculate and be picked up by undamaged Kupffer cells in the sinusoids, this would have resulted in occurrence of Kupffer cells showing evidence of being in early uptake phases between 1 and 12 hours after the injection, but was not observed (*b*) The electron microscopic observations did not reveal signs of degeneration or necrosis of the Kupffer cells, neither were pictures obtained suggesting that these cells were detaching from the sinusoids (*c*) Images indicating that sinusoidal lining cells were dividing or proliferating during the period between 1 and 24 hours after the injection of Neosilvol® were not encountered

The ultimate and most likely explanation for the disappearance of the large silver iodide granules from the Kupffer cell lysosomes would therefore seem to be that these large molecule aggregates were broken down into single silver iodide molecules within the lysosomes The molecular weight of silver iodide (235) is compatible with escape of the molecules through intact lysosomal (and plasma) membranes (16) The occurrence of silver in the parenchymal cells of the liver —as revealed by preliminary results of atomic absorption studies (1)—coincided with disappearance of the large silver iodide macromolecules from the Kupffer cell lysosomes Furthermore, presence of a finely granular material was observed in the lysosomes of the parenchymal cells simultaneously with the demonstration of accumulation of silver in these cells (1) Taken together, the above data and considerations favour the theory that 200–1 300 Å large aggregates of silver iodide molecules sequestrated in Kupffer cell

Fig 12 Neosilvol® intravenously, 48 hours Portions of two parenchymal cells (peribiliary regions) with numerous bodies and vacuoles containing electron dense, finely granular material One of these vacuoles (V) is shown at higher magnification in the *inset* It is clear from the inset that the granular material is composed of much smaller elements than the silver iodide particles illustrated in Figures 8–11 OsO_4, Epon section staining with lead citrate × 17,800, inset × 52,800

Fig 13 Neosilvol® intravenously 72 hours Portion of Kupffer cell with many lysosome like bodies of variable size There are no large typical silver iodide particles present in these bodies However some of them (arrows) appear to contain a finely granular material OsO_4, Epon section staining with lead citrate ×17,500

lysosomes (and presumably the lysosomes of other "reticuloendothelial cells") are broken down into single molecules capable of escape through intact lysosomal and plasma membranes, these small molecules released from the Kupffer cells might reach the parenchymal cells either directly via Disse's space or indirectly by uptake from the blood circulating through the sinusoids

The ultimate goal of the present investigation was to try to achieve a selective uptake of dense macromolecules in Kupffer cells, hereby defining the most favorable conditions for attempts to isolate Kupffer cell lysosomes from homogenates of rat liver The findings indicate that loading with Thorotrast® is not a particularly useful method for this purpose due to the fact that the uptake is not limited to Kupffer cells except during a very brief period of time immediately after the injection of the compound On the other hand, the large silver iodide macromolecules were restricted to Kupffer cell lysosomes, hence, pretreatment of animals with an intravenous injection of Neosilvol® should be a useful approach to the isolation of Kupffer cell lysosomes However in order to obtain optimal conditions for isolation, homogenization ought to be performed within the first few hours after the injection since otherwise—at later intervals—loss of macromolecules might have occurred with possible resultant accumulation of silver-containing breakdown products in parenchymal cell lysosomes At 24 hours and later one can expect to be able to isolate a lysosomal fraction with properties similar to those of iron loaded lysosomes since at these late intervals both parenchymal and Kupffer cell lysosomes may show increased density

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The method for the atomic absorption measurements of silver was worked out by Dr *C U Wetlesen* at Sentralinstitutt for Industriell Forskning, Oslo Norway where also the assays were performed We are greatful indebted to Dr *Wetlesen* for his valuable aid and cooperation

The technical assistance of Mrs *Silva Mengarelli* Mrs *Britt Marie Akerman* and Mr *Magnus Norman* is also gratefully acknowledged

This work was supported by grants from the *Swedish Medical Research Council* (Project number B74 12X 1006-09C) and from the *Norwegian Council for Science and the Humanities*

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RENIN INACTIVATION *IN VITRO* IN PREGNANT AND POST PARTUM RABBIT UTERINE AND KIDNEY TISSUE

The Mechanism of the Rapid Pronounced Decrease in Uterine Renin Content post partum in Rabbits II

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In slices of pregnant and post partum rabbit uterus 80 per cent of the endogenous renin was gradually inactivated during incubation *in vitro* at 37° C for 44 hours In slices of rabbit kidney only 30 per cent was inactivated Contrary to this there was only a slight, inconsistent, decrease in renin content in similarly incubated homogenates and extracts of uterine and renal tissue The results show that intact tissue from both the kidney and especially the uterus have a capacity for inactivation of renin

In the accompanying paper it was demonstrated that the rapid pronounced fall in rabbit uterine renin content post partum could not be explained by a release of the renin to the blood This was concluded because the post partum peripheral plasma renin concentration was measured to be within the non-pregnant range, while very high renin levels would have been expected if release of greater amounts of the uterine renin to the blood had occurred This finding suggested that local factors were involved in the post partum inactivation of uterine renin

The aim of the present study was to investigate whether or not this inactivation of uterine renin in the post partum period could be demonstrated under *in vitro* conditions

For this purpose the renin content in intact tissue *slices from the uterine wall* (and for comparison kidney cortex) from pregnant and post partum rabbits was measured before and after incubation at 37° C for different periods of time (part I A) It was further measured whether or not the renin content of the supernatant in the incubation mixture forms a constant percentage of the total renin content throughout the incubation (part I B)

Furthermore the renin content was determined after incubation for different periods of time of either the *homogenates* of uterine and kidney tissue (part II) or the *supernatants from suspensions of slices which previously had been incubated* for zero or 2 hours (part III)

Received 27 vi 74 Accepted 27 vi 74

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MATERIAL AND METHODS

Animals

Uterine wall and renal cortex from 3 pregnant (26th-31st day of pregnancy) and 5 post partum (about 10 33 hours after delivery) albino country rabbits from the State Serum Institute, were used

Part I A Renin Content of Tissue Slices

All operations were performed under anaesthesia with pentobarbital (40 mg per kg body weight i.v.), in some cases supplemented with ether Uterine horns and kidneys were removed aseptically by laparotomy, sterile utensils being employed during the whole preparation For the tissue preparation an antimesometrial strip of all layers of the uterine wall or a piece of renal cortex was used Blood was as far as possible removed from the tissue by washing in sterile 0 9 per cent NaCl followed by a short removal of the fluid by blotting on filter paper or (in 2 of the pregnant animals) by passing the tissue along the surface of several dry Petri dishes Using small scissors the tissue was cut into maximally 3 mm small pieces under careful mixing Portions of cut tissue weighing 200–300 mg were transferred to (3 × 3 cm) test tubes with lids The tissue was then further cut to smaller pieces measuring maximally about 1 5 mm The amount of tissue in a test tube was determined by weighing To each tube 2 5 ml of incubation buffer and 100 µl of neomycine solution was then added These preparations which lasted 45 60 min were performed at room temperature

Usually 24 such samples were prepared from each organ, and these were incubated for 0, ⅓, 2, 7, 20 and 44 hours in quadruplicate

Incubation buffer was in all cases NaCl 119 mM, $NaHCO_3$ 17 5 mM, KCl 7 0 mM, $CaCl_2$ 2 0 mM, $MgSO_4$ 12 0 mM, Glucose 11 0 mM and NaH_2PO_4 1 2 mM Before use the buffer was sterilized by passage through a Millex filter (pore size 0 22 µ) and equilibrated with 95 per cent O_2 5 per cent CO_2 pH was 7 4

Neomycine solution was neomycine sulphate 60 mg/ml of incubation buffer, adjusted to pH 7 4 with 5N NaOH and sterilized by passage through a Millex filter (pore size 0 22 µ)

Incubation the tubes were incubated at 37° C without lids in a Dubnoff Metabolic Shaking Incubator and aerated with about 30 l sterile 95 per cent O_2–5 per cent CO_2 per hour The fluid in the incubator was distilled water containing 1 per mille Rodalon® (in the case of the tissue preparations from 2 of the pregnant animals no Rodalon® was added) Evaporation from the samples during incubation was 4 per cent per 24 hour, as measured by weighing After incubation the samples were placed in an ice bath, the pH in the supernatant was measured, and the carbon dioxide driven off by addition of 2N HCl to a pH of 6 0–6 5 The samples were then frozen at 20° C until extraction and assay

pH in the supernatant of the samples after incubation remained rather stable about 7 4 in nearly all incubations In one case in which the uterus was removed on the 31st day of pregnancy, in two cases 10 hours and in one 26 hours after parturition, pH decreased to values about 6 2 These changes in pH were not followed by any change in the degree of inactivation of renin

Bacteriological control from 26 samples incubated for 0, 7 or 20 hours, a few drops of the tissue buffer supernatant were used for bacteriological investigation None of them gave bacterial growth

Part I B Renin Content in Supernatants

For determination of the renin content in the supernatants during incubation, 400 µl supernatant of the tissue buffer mixture was removed after different incubation periods and frozen separately after a centrifugation at 3000 *g*

Part II Tissue Homogenates

After removal of the blood from the tissue, as in section I, the tissue was homogenized in a Potter Elvehjem homogenizer after addition of incubation buffer, giving a dilution of the tissue of 1 3 to 1 11 The preparations were performed at 0° C and lasted up to 3 hours After addition of 100 µl neomycine solution samples of 2 6 ml were incubated for 0, ⅓, 7, 20 or 44 hours as in section I pH in these samples was 7 0–7 8 after the incubation Hog renin added to homogenate was from the Nutritional Biochemical Corporation (Cleveland)

Part III Supernatants from Suspensions of Slices

Non incubated and 2 hours incubated uterine or kidney slices in buffer were centrifuged at 3000 *g* for 10 min The supernatants were incubated in open tubes as in section I for 7 or 20 hours After incubation pH in these samples was 7 4 7 7 Before incubation 80 µl aliquots of each sample were removed and 10 µl 3 M Tris HCl pH 7 4 was added to keep a pH of 7 4 during storage These samples were used for determination of the initial renin concentrations

Tissue Extraction

Incubated tissue slices (part I A) and homogenates (part II) were frozen and thawed 3 times and then homogenized in a Potter Elvehjem homogenizer after addition of 2 5 ml pyrophosphate buffer (0 02 M pH 5 3) After about further 18 hours at 4° C, the samples were centrifuged at 3000 *g*, and the supernatants stored at –20° C until assay

Renin radioimmunoassay was performed according to the principle of *Poulsen* (1971) using trapping of Angiotensin I (*Poulsen & Jørgensen* 1974) The renin concentration was expressed in Goldblatt units by reference to a purified hog renin

standard preparation*, which in a series of dilutions was included in each assay

The reproducibility of the assay from day to day was determined by repetitive assays of the same uterine extract, which (n = 21) showed a SD of ± 10 per cent Usually the 24 samples of one tissue incubation were analysed together in the same series, giving a somewhat higher precision

RESULTS

Part I A The Total Renin Concentration in Slices plus Media during Incubation

a Pregnant Uterus

During the first 7 hours of incubation only a slight loss of renin, if any, was observed (Fig 1, open symbols) in four out of the five experiments In the last case there seems to be a greater loss of renin at the beginning of the incubation Upon further incubation the total content of enzymatically active renin in slices plus supernatant decreased to about 50 per cent and 20 per cent of the initial amounts after 20 and 44 hours of incubation, respectively No systematical differences were observed when tissue from 26th, 27th and 31st day of pregnancy were used

The *initial renin concentration* varied between 12 and 34 GU per g of tissue However, this difference did not influence the relative decrease in renin content during incubation

The *half life* for renin was determined for the experiments in Fig 1 A plot of the logarith for the renin concentration versus incubation time was constructed for each experiment Provided that no renin formation occurred during incubation and that the renin inactivation process was of first order a straight line would be obtained The apparent half life for renin varied however with the incubation period

Within the first 7 hours of incubation the half lives appeared to be between 17 and ∞ hours (mean 45 hours, calculated from the regressions) In no cases were the coefficients of correlation significantly different from zero ($p > 0.05$) A significant destruction of renin took place, however, after 7 hours of incubation Between 7 hours the half life for renin was 14–26 (mean ı hours and between 20–44 hours the half life 14–21 (mean 17) hours In both cases the cients of correlation were significantly dil from zero ($p < 0.01$)

b Post partum Uterus

There was a decrease in renin (Fig closed symbols) in all cases (except perha one) from the beginning of the experim continuing at a rather constant rate for tl first 20 hours after which the rate decreas After 2 and 7 hours there was a decrease about 85 and 66 per cent respectively of th original content After 20 and 44 hours th decrease was to about 30 and 20 per respectively of the initial amount

The *initial renin concentrations* varied be tween 3 6 and 33 GU per g tissue, with t lowest values found in the uteri, which wer removed latest in the post partum peri This difference had no apparent influen on the relative decrease in renin, giving l differences in the absolute amounts of ren inactivated during the incubation

The semilogarithmic plots of renin versus i cubation time gave *half lives for renin* for the 0– and 7–20 hour incubation periods of 11–18 (mear 13) and 7–17 (mean 11) hours with all coefficient of correlation significantly different from zerc ($p < 0.01$ and in one case $p < 0.05$) In tl 20–44 hour incubation period half lives of 23 hours to ∞ (mean 37 hours) were found in most cases with lines of regression significantly different from 0

c Kidney Cortex from Pregnant and post partum Rabbits

In both cases there was a slow decrease in renin in slices plus supernatant during the incubation (Fig 2) At 20 and 44 hours there was a fall to about 85 and 70 per cent of the initial renin content

Because of the slow decrease in renin and the larger scatter in the individual experiments, only the regression lines for the whole incubation periods were calculated These gave *half lives* between 56 and 470 hours (mean 91 hours) with coefficients of correlation except for one case significantly different from 0 ($p < 0.05$)

* Kindly supplied by Dr Haas and identical with the preparation donated by Dr Haas to the National Institute for Biological Standards and Control, Holly Hill, Hampstead London

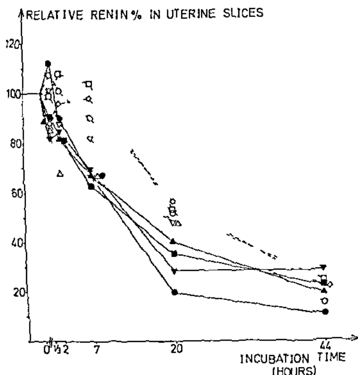


Fig 1 The decrease in renin content with time in uterine tissue incubated in vitro Slices of uterus from pregnant rabbits (open symbols) and the post partum period (closed symbols) were incubated in vitro Each symbol represents a single uterine horn each point being the mean of quadruplicate incubations (S E M ± 8 per cent) Uterus was removed either on the 26th (△▽) (both horns), 27th (□◇) (both horns) and on the 31st day (○) of pregnancy, or after delivery about 10 h (▲) 10 h (▼), 26 h (●) and 33 h (■) post partum The initial renin concentrations were in absolute values (same sequence) 27, 30, 31 34, 12 22, 33, 15 and 3 6 GU per g of tissue

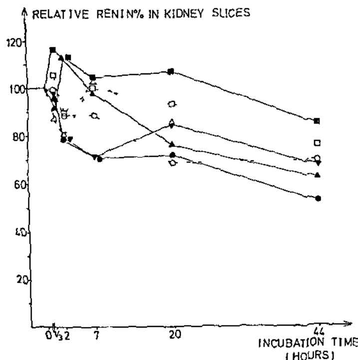


Fig 2 The change in renin content with time in kidney tissue incubated in vitro Slices of kidneys obtained simultaneously with uterus were incubated in vitro Symbols as in Fig 1 The initial renin concentrations were in absolute values (△) 21, (□) 7 5 (○) 18 (▲) 11, (▼) 16 (●) 27 and (■) 17 GU per g of tissue

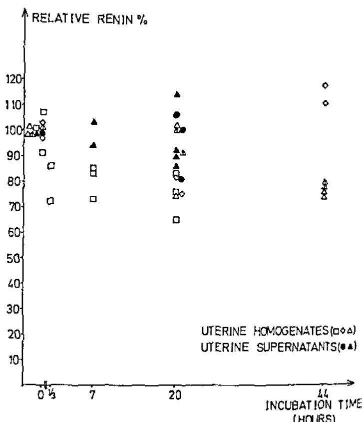


Fig 3 The renin content in uterine homogenates and *isolated supernatants* with time, when incubated at 37°C The points give the relative renin content after incubation (□, ◇ and △) *homogenates* from tissue removed about 18, 26 and 33 hours post partum and diluted 1 11, 1 3 and 1 5, respectively (● and ▲) *supernatants* from tissue slices prepared on the 31st day of pregnancy and 10 hours post partum respectively ▽ indicates preparation △ with added exogenous hog renin The initial renin concentrations were in absolute values (□) 0 8, (◇) 2 8, (△) 0 4, (▽) 1 45, (●) 0 17 and (▲) 0 33 GU per ml

Part I B Renin Concentration in Supernatants of the Tissue Slices

a Pregnant Uterus

The renin concentration in the supernatant was rather constant during incubation However, the relative amounts of renin present in the supernatant were between 5 and 12 per cent of the total amount of renin present during the first 0–20 hours of incubation, rising to about 65 per cent (mean of only 2 estimations) at 44 hours

b Post partum Uterus

Again the concentration of renin in the supernatant was rather constant during incubation The *relative* amounts of renin in the supernatant, however were 5 10 per cent of the total at the beginning of the experiments and rose gradually during the incubation At 20 hours it was 20–70 per cent with most values being about 25 per cent and at 44 hours between 20 and 100 per cent with most values about 90 per cent

c Pregnant and post partum Kidney

The renin concentration in the supernatant rose gradually during incubation The relative amounts of renin in the supernatant rose gradually from 1–6 per cent of the total at the beginning of the experiments to about 40 per cent at 44 hours

Part II Incubation of Homogenates

From 3 *uteri* removed about 18, 26 and 33 hours post partum, homogenates in dilutions of 1 11, 1 3 and 1 5, respectively were incubated for up to 44 hours During the incubation there was no, or only a slight (maximally 25 per cent) decrease in renin (Fig 3 open symbols) In one experiment hog renin was added to the original concentration of 0 4 GU × ml⁻¹ to give a final concentration

of 1 45 GU × ml⁻¹ The exogenously added renin was inactivated at the same rate as the endogenous In a single experiment with homogenized tissue from a *kidney*, removed about 18 hours post partum, the loss of renin was 20 per cent at 20 hours

Part III Incubation of Supernatants from Suspensions of Slices

When supernatants from pregnant and post partum *uterus slices* were incubated separately there was no significant loss of renin during a 20 hours incubation (Fig 3, closed symbols) The concentration of renin after 20 hours incubation was 95 6 ± 4 4 per cent (mean ± SEM, n = 7) of the initial

In similar incubations of supernatants from pregnant and post partum kidney the content of renin after 20 hours incubation was 105 6 ± 3 0 per cent (mean ± SEM, n = 7) of the initial

DISCUSSION

The rapid pronounced fall in uterine renin concentration post partum to about non-pregnant levels within 2 days (*Bing & Faarup* 1968) is much faster than the decrease in uterine weight, which is about 50 per cent in the first 2 days implying that the inactivation of renin differs from the general involutional changes Release of renin from uterus to the blood was not found to play any significant role in this inactivation, as the peripheral plasma concentration of renin in the early post partum period is not higher than in non pregnant rabbits (*Jorgensen* 1975)

In *in vitro* experiments with crude preparations as performed in this study, any concentration determined after incubation may be the result of simultaneous formation and inactivation of renin

In the present study a loss of 80 per cent of the initial renin content was seen in slices of pregnant and post partum *uterine* slices incubated for 44 hours In slices of *kidneys* from the same animals a loss of 30 per cent was seen

The relative renin inactivation was independent on 10-fold variations in the starting renin concentration This may reflect a I order rate inactivation

Such a marked inactivation of renin could neither be found in *homogenates* of uterus, giving maximally 25 per cent loss nor in *isolated supernatants* from suspensions of slices of uterus or kidney where no significant loss of renin occurred

These results indicate that pregnant and post partum *uterine* tissue have a considerable ability to inactivate renin That this inactivation was not found to the same extent in homogenates and not found at all in isolated supernatants could indicate 1) that the inactivation required "vital cells", 2) that the process took place in organelles where special conditions of environmental are fulfilled, or be due to 3) a simple matter of concentration, or 4) release of an inhibitor from organelles during homogenization

The slow but also significant decrease in renin seen in incubated slices of *renal* c[illegible] is consistent with the direct role of the k[illegible] in inactivation of circulating renin *in* [illegible] which has been demonstrated by *Hou[illegible] et al* (1942) and *Peters-Haefeli* (1971)

This work was supported by [illegible] *Danish Medical Research* [illegible] *Heart Association* and *Carl* [illegible]*og* [illegible] (to *Jørgen Jørgensen*) and [illegible] *Foundation* (to *Jens Bing*)

The author wishes to thank [illegible] *sen* M D for valuable assistance [illegible] logical investigations, *Knud* [illegible] valuable discussion and Mrs [illegible] *Jane Czuba* and Miss *Ragna J*[illegible] ble technical assistance

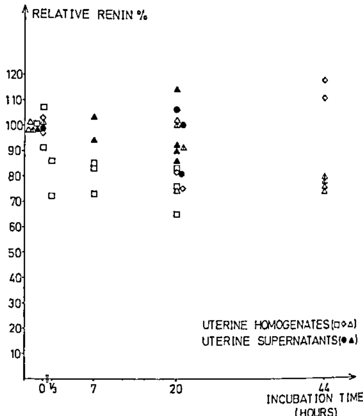
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Jørgensen, J Concentrations of renin

Fig 3 The renin content in uterine homogenates and *isolated supernatants* with time, when incubated at 37° C The points give the relative renin content after incubation (□, ◇ and △) *homogenates* from tissue removed about 18, 26 and 33 hours post partum and diluted 1 11, 1 3 and 1 5, respectively (● and ▲) *supernatants* from tissue slices prepared on the 31st day of pregnancy and 10 hours post partum, respectively ▽ indicates preparation △ with added exogenous hog renin The initial renin concentrations were in absolute values (□) 0 8, (◇) 2 8, (△) 0 4, (▽) 1 45, (●) 0 17 and (▲) 0 33 GU per ml



Part I B Renin Concentration in Supernatants of the Tissue Slices

a Pregnant Uterus

The renin concentration in the supernatant was rather constant during incubation However, the relative amounts of renin present in the supernatant were between 5 and 12 per cent of the total amount of renin present during the first 0–20 hours of incubation rising to about 65 per cent (mean of only 2 estimations) at 44 hours

b Post partum Uterus

Again the concentration of renin in the supernatant was rather constant during incubation The *relative* amounts of renin in the supernatant, however were 5 10 per cent of the total at the beginning of the experiments and rose gradually during the incubation At 20 hours it was 20 70 per cent with most values being about 25 per cent and at 44 hours between 20 and 100 per cent with most values about 90 per cent

c Pregnant and post partum Kidney

The renin concentration in the supernatant rose gradually during incubation The relative amounts of renin in the supernatant rose gradually from 1–6 per cent of the total at the beginning of the experiments to about 40 per cent at 44 hours

Part II Incubation of Homogenates

From 3 *uteri* removed about 18, 26 and 33 hours post partum, homogenates in dilutions of 1 11, 1 3 and 1 5, respectively were incubated for up to 44 hours During the incubation there was no, or only a slight (maximally 25 per cent) decrease in renin (Fig 3 open symbols) In one experiment hog renin was added to the original concentration of 0 4 GU × ml^{-1} to give a final concentration

Acta path microbiol scand Sect A, 82 767-776, 1974

STREPTOZOTOCIN-DIABETES IN THE CHINESE HAMSTER

Long-Term Effects on the Light Microscopic Structure of the Pancreatic Islet Tissue, Liver and Kidney

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One day after injection of 200 mg streptozotocin/kg body weight in Chinese hamsters, necrotic cell debris or marked degenerative changes were seen in the pancreatic islets light microscopically Both β, α_2- and α_1 cells seemed to be affected During the first weeks, in particular, signs of regeneration and neoformation of islet tissue were evident in the pancreatic tissue Labelling with ^3H thymidine revealed an increased incorporation of this isotope in islets of diabetic animals Tubular structures and proliferating buds consisting mostly of 'clear cells' were seen 'Clear cells' were also observed sometimes in aggregates in islets with degenerative changes The high incorporation of labelled thymidine at the same time as 'clear cells" were observed in the islet tissue suggest that these cells are involved in islet cell regeneration After one week, up to about one month, large islets containing hypertrophied cells with a large nucleus and a prominent nucleolus were found Hydropic degeneration and even necrosis sometimes occurred in these islets The regenerative activity as well as the hypertrophic islets disappeared at the end of the observation period (3 months) at which time relatively small islets with degranulated β cells and irregularly distributed α - and α_1-cells with variable staining properties and nuclear sizes were seen

The effect of the diabetogenic substance streptozotocin on the blood glucose and the volume and diameter of the pancreatic islets in Chinese hamsters has been described in a previous report (*Wilander* 1974), as well as light and electron microscopic changes in the pancreatic islet tissue in the first 24 hours after injection (*Wilander* & *Boquist* 1972), It has been shown that streptozotocin exerts a direct cytotoxic effect on the pancreatic islet β cells and even α_1 cells The streptozotocin injected animals either show a hyperglycaemic phase 24 hours after injection, with return to normal blood glucose values within three days, or in most animals a persistent hyperglycaemia with no signs of reversal

The aim of the present investigation was to study the long term effect of streptozotocin on the light microscopic picture of the pancreatic islet tissue, liver and kidneys in Chinese hamsters, mainly for comparison with previously described blood glucose variations (*Wilander* 1974) as well as with the light microscopic pancreatic islet structure in alloxan-diabetic (*Boquist* 1968) and spontaneous hereditary diabetic (*Meier* & *Yerganian* 1959, *Carpenter et al* 1967, *Boquist* 1969 and *Carpenter et al* 1970) animals of the same species

Received 6 v 74 Accepted 20 vi 74

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Non diabetic Chinese hamsters of both sexes were used, partly the same animals as those referred to in earlier reports (*Wilander* & *Boquist* 1972, *Wilander* 1974) They were 4–14 months old, weighed 17 39 gm and were selected at random from litters in which no cases of overt diabetes had occurred for at least six generations The animals were free from hyperglycaemia and glucosuria before the start of the experiment

One hundred non fasting Chinese hamsters were injected intraperitoneally with 200 mg streptozotocin/kg body weight The streptozotocin was dissolved in citrate phosphate buffer at pH 4 0 and a concentration of 2 per cent Eighteen control animals were given a corresponding volume of plain buffer intraperitoneally

Five streptozotocin injected animals and two buffer controls were injected with ^{3}H thymidine (spec act 5 Ci/mM) intraperitoneally in a dose of 1 μCi/gm body weight 8 times from the first to the fourth day after injection of streptozotocin

Blood samples were taken 1, 3, 6, 13, 20, 27, 55 and 83 days after the injection of streptozotocin or buffer solution and blood glucose determinations were performed by the glucose oxidase method, using a commercially available kit (AB Kabi Stockholm, Sweden) Clinistix® (Ames Co London, England) was used to test for glucosuria

Twenty five diabetic animals died spontaneously during the experimental period The rest were sacrificed 1 2 3 4 6 10 11 13 20 27, 55 and 83 days after the injection The ^{3}H thymidine injected animals were killed on the fourth day Specimens from each sacrificed animal were taken from the pancreas liver and kidneys Those from the pancreas were fixed in Bouin s fluid, dehydrated, embedded in paraffin sectioned in 4 μ thick sections and stained with van Gieson s stain aldehyde fuchsin sometimes counterstained with ponceau fuchsin (*Hultquist* 1962) chrome alum haematoxylin toluidine blue (1 per cent pH 7 0) alkaline Congo red (*Puchtler et al* 1962) and silver impregnations according to *Hellerstrom* & *Hellman* (1960) and *Grimelius* (1968) From the ^{3}H thymidine injected animals sections from the pancreas were taken for a micro-autoradiographic study using the conventional liquid emulsion method (Ilford K2 diluted 2 1 with redistilled water 45° C) After exposure and development the sections were stained with Ehrlich s haematoxylin Specimens from the kidneys and liver were fixed in 10 per cent formalin and stained with van Gieson s stain and haematoxylin-eosin Specimens from the kidneys were also stained with Periodic Acid Schiff stain

RESULTS

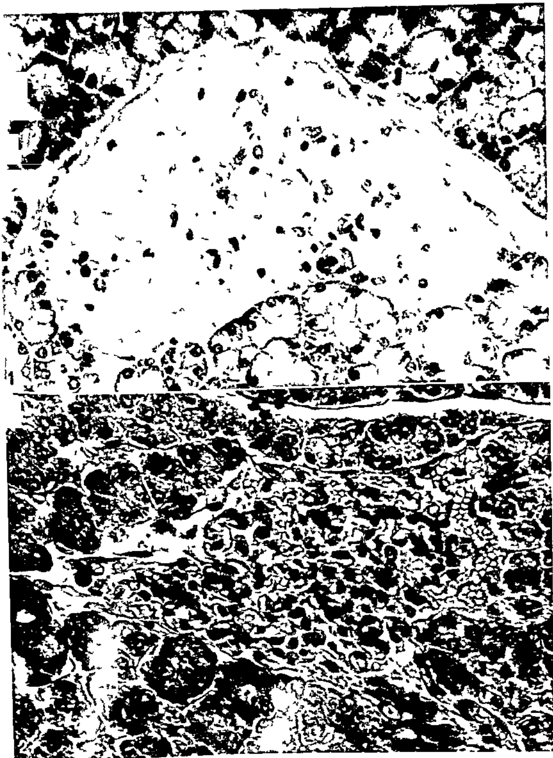
Blood glucose The result of the blood glucose analyses has been described in detail in an earlier report (*Wilander* 1974) Shortly, there was a marked hyperglycaemic peak (about 350 mg/100 ml) one day after the injection of 200 mg/kg body weight of streptozotocin. During the following days and months moderately elevated values were recorded (about 200 mg/100 ml) in most animals From the third day to the end of the observation period there were no signs of a decrease in blood glucose towards normal values in the diabetic animals All diabetic animals with hyperglycaemia also showed glucosuria The buffer injected hamsters were normoglycaemic

Pancreatic Islet Tissue

Degenerative changes Twenty four hours after the injection of streptozotocin there was almost total destruction of the endocrine pancreas in most animals No β , α - or α_1-cells could be found The islets contained only capillary endothelial cells and a few inflammatory cells surrounded by an amorphous material (Fig 1), but the changes varied within the same pancreas and between different animals In some islets, the degenerative changes were less pronounced In these islets the β cells showed an irregular pyknotic nucleus and vacuolization and the staining properties of their cytoplasm varied (Fig 2) Irregular α- and α_1-cells were also seen in these islets, often with only slight granulation Mitoses were sometimes observed in the few remaining cells of the nearly totally destroyed islets as well as in those with marked

Fig 1 Pancreatic islet in a Chinese hamster 24 hours after the injection of streptozotocin There is total destruction of the endocrine cells The islet contains amorphous material, capillary endothelial cells and a few inflammatory cells Van Gieson, × 400

Fig 2 Pancreatic islet in a Chinese hamster 3 days after injection of streptozotocin Degenerative endocrine cells with nuclear pyknosis and cytoplasmic vacuolization are seen Aldehyde fuchsin × 400



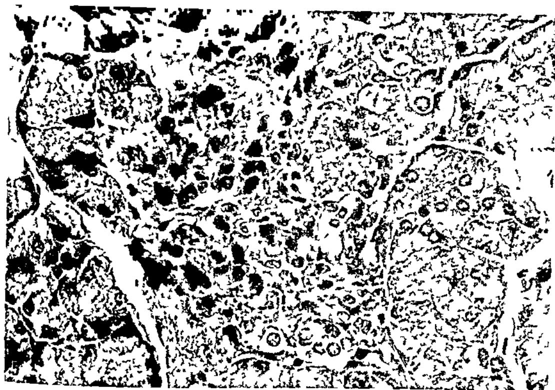


Fig 5 Pancreatic islet in a Chinese hamster 5 days after injection of streptozotocin Clear cells intermingle with the degenerative endocrine cells Aldehyde fuchsin × 400

degenerative changes In the buffer injected animals the islet tissue appeared normal

Regenerative changes During the first month but mainly during the first and second weeks there were signs of regeneration and neoformation of islet tissue with tubular structures either lying within islet tissue or unrelated to it Clear cells could be seen to proliferate from these structures (Fig 3) Aggregates of clear cells were also seen in the exocrine pancreas without obvious association with tubular structures (Fig 4) as well as in islets with degenerative changes (Fig 5)

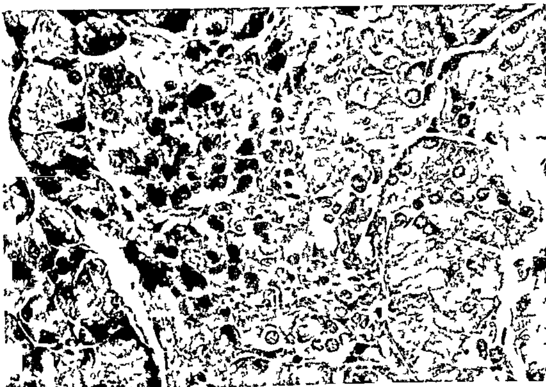
In the diabetic animals islets were relatively rarely found in sections from the pancreas but in the few islets observed there was a high incorporation of ^{3}H thymidine after injection of this isotope (Fig 6) Labelled cells were found both in the centre and in the periphery of the islets but could not be identified with certainty In buffer injected animals, labelled islet cells were occasionally seen (Fig 7)

The degree of regeneration of islet tissue varied in different animals In several animals there were no or only slight signs of neoformation of islet tissue The regenerative activity declined during the experimental period At the end of this period no signs of regeneration could be found except for the occasional presence of 'clear cells' in the islets

Fig 3 Pancreatic tissue in a Chinese hamster 11 days after injection of streptozotocin Tubular structures with proliferating buds containing clear cells are seen The tubular structures are surrounded by mononuclear inflammatory cells Aldehyde fuchsin × 640

Fig 4 Pancreatic tissue in a Chinese hamster 11 days after injection of streptozotocin In the exocrine pancreas there is an islet like cluster of large pale, not granulated cells These show no clear relationship to tubular structures or mature islets Aldehyde fuchsin × 960





F g 5 Pancreat c let n a Ch nese ham ter 5 days af er nject on of streptozotoc n Clear cells nter m ng e w th the degenerati e endocrine cells Aldehyde fucl s n × 400

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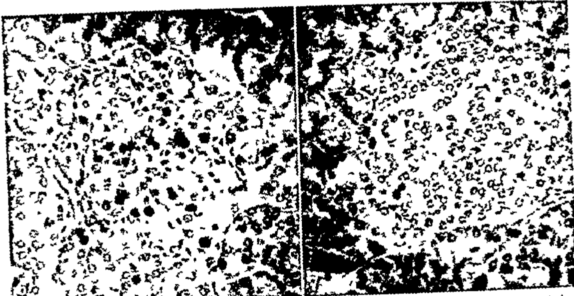


Fig 6 Micro-autoradiograph of ^{3}H thymidine labelled pancreatic islet in a Chinese hamster 4 days after injection of streptozotocin exhibiting an increase in labelled cells Ehrlich s haematoxylin, × 400

Fig 7 Micro-autoradiograph of ^{3}H thymidine labelled pancreatic islet in a normal control showing a few labelled cells in the periphery of the islet Ehrlich s haematoxylin × 400

Small aggregates of lymphocytes were seldom seen in the normal pancreas These seemed to increase in number in the diabetic animals mainly in association with tubular structures and clear cells (Fig 3)

Hydropic degeneration Large islets with hydropic degeneration were found in the pancreatic tissue in streptozotocin treated Chinese hamsters Islets of this kind were seen in some cases after about one week and in all diabetic animals after one month after which time they gradually disappeared They consisted of hypertrophied cells with a large nucleus and a prominent nucleolus (Fig 8) Especially in their peripheral area the cells often showed marked hydropic degeneration sometimes obviously resulting in necrosis and fragmentation preceded by pronounced nuclear polymorphism and hyperchromatism Tubular structures and aggregates of lymphocytes were occasionally seen in association with these islets The cytoplasm of the endocrine cells in the periphery of these islets failed to stain with silver impregnations but was often slightly positive to porcine fuchsin stain A few weakly aldehyde fuchsin cells were seen in the centres It is possible that many of the endocrine cells with hydropic degeneration in the peripheral areas of the islets were α_1 cells

Additional findings On the whole the islets appeared smaller in the diabetic animals than in the normal controls Further the nucleus varied in size and often seemed larger in the β cells as well as in the α cells in the streptozotocin injected animals (Fig 9) The β-cells were weakly stained The α-cells were irregularly distributed in the islets and showed great variation in staining properties compared with normal controls Essentially

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Fig 8 A large pancreatic islet in a Chinese hamster 11 days after injection of streptozotocin It consists of hypertrophied cells with a large nucleus a prominent nucleolus and hydropic degeneration in the periphery Aldehyde fuchsin × 400

Fig 9 Pancreatic islet in a Chinese hamster 55 days after injection of streptozotocin The islet cells show varied nuclear sizes and often a distinct nucleolus as signs of increased activity Van Gieson × 640

normal α_1 cells were found in the peripheral areas of the islets in the diabetic animals.

No amyloid deposits were observed either in the experimental animals or in the normal controls

Liver Structure

Fatty vacuolization was found in the liver in about 50 per cent of the diabetic animals 24 hours after the injection of streptozotocin It was less pronounced after three days and disappeared during the following days After one month there were in a few diabetic animals liver cell nuclear polymorphism and proliferation of tubular structures in the portal regions These phenomenon were observed in about half of the experimental animals after three months No alterations of the above mentioned kind were observed in the liver tissue of normal controls

Renal Structure

No obvious changes in the kidneys were seen in normal controls during the experimental period After three days and during the following days and months tubular dilatation and degenerative changes in the tubular epithelium were observed in the streptozotocin injected animals sometimes with small accumulations of glycogen These changes were more pronounced at the end of the observation period at which time they were evident in almost all diabetic animals No glomerular or appreciable tubular necrosis was found There was also some dilatation of the renal pelvis at the end of the observation period in the diabetic animals

DISCUSSION

This study confirms the observations made in a previous investigation (*Wilander & Boquist* 1972) that streptozotocin acts as a β-cytotoxic substance in Chinese hamsters causing necrosis or marked degenerative changes in the β-cells with nuclear pyknosis and cytoplasmic vacuolization Degenerative changes and necrosis were also found in the α ... findings which have also been made in the rabbit (*Lazarus & Shapiro* 1972), but in no other laboratory animal hitherto examined. There seems to be a difference in the cytotoxic action of alloxan and streptozotocin in the Chinese hamster as degenerative changes without obvious necrosis in the β cells together with damage to α-cells have not been observed after administration of alloxan (*Boquist* 1968).

Tubular structures with proliferating buds of "clear cells" as signs of regeneration and restoration of the pancreatic islet tissue to normal in alloxan diabetic mice (*House* 1958 *Bunnag et al* 1967) and in alloxan diabetic Chinese hamsters (*Boquist* 1968) have been described In streptozotocin diabetic rats *Arison et al* (1967) found only slight structural signs of regeneration and *Steiner et al* (1970) claimed that β-cell regeneration from insular cells or islet neoformation from extra insular cells played no significant role after streptozotocin injection in the same animal *Logothetopoulos & Brosky* (1968) observed significant mitotic activity in the early phase after streptozotocin injection in mice This activity was much less marked than after injection of alloxan In the diabetic mutant mouse, an increased incorporation of ^{3}H thymidine in the β-cells has been observed in the prediabetic phase, during the onset of early hyperglycaemia and in animals with established hyperglycaemia, during the terminal phase of this syndrome, however, labelled endocrine cells were infrequent (*Like & Chick* 1970) The results obtained in this study are mainly in accordance with observations made in the diabetic mutant mouse and streptozotocin diabetic mice Although there were obvious signs of neoformation of islet tissue especially during the early phase after streptozotocin injection, this seemed to be insufficient to restore the islet tissue to normal Islet neoformation in streptozotocin diabetic Chinese hamsters seems to take place through mitoses of endocrine islet cells and probably also through cell proliferation from tubular structures The occurrence of "clear cells" in a period of increased incorporation of labelled

hymidine in islet tissue indicates that these ells may participate in islet cell regeneration The difference between degranulated β cells and 'clear cells' has been pointed out by *Edström* (1972)

The persistent marked islet atrophy which has been observed in streptozotocin diabetic Chinese hamsters (*Hellander* 1974) is worthy of note since a decrease in islet cell volume and a decrease in β cell mass also characterize Chinese hamsters with spontaneous hereditary diabetes (*Carpenter et al* 1967), suggesting that these alterations are a primary aetiologic mechanism in the production of the diabetic syndromes These observations may also be of some importance with respect to the aetiology of human juvenile diabetes mellitus since a decrease in β cell mass in this condition (*Gepts* 1965, *Maclean* & *Ogilvie* 1955 and 1959) has also been described In Chinese hamsters with spontaneous hereditary diabetes β cell destruction but no signs of islet cell regeneration is observed This may be a fact of decisive significance in the pathogenesis of diabetes mellitus in this animal species (*Boquist* 1969) However whether the islet atrophy in human juvenile diabetes mellitus is a result of increased destruction of β-cells or defective neogenesis of these cells or a combination of the two is not clear

During the first month and especially during its later part, large islets consisting of endocrine cells with a large nucleus a prominent nucleolus varying degrees of vacuolization in the cytoplasm and also swelling were seen Necrosis of what at least may have been partly α cells was also found in the periphery of these islets An increase in nuclear size suggests an increased protein synthetic activity (*Hultquist* 1962 *Oehlert* & *Schultze* 1960) in the present investigation sometimes probably resulting in exhaustion An increase in mean nuclear diameter in β cells and α cells of streptozotocin diabetic rats (*Steiner et al* 1970) and in β and α_1 cells of streptozotocin treated guinea pigs *Petersson et al* (1970) has been reported *Katsilambros et al* (1970) found elevated levels of serum glucagon in rats after streptozotocin injection Likewise *Unger et al* (1970) demonstrated strikingly elevated plasma glucagon levels in diabetic patients with severe keto acidosis The mechanism underlying the increase in α cell activity remains unclear

The fatty metamorphosis of liver cells observed in several Chinese hamsters 24 hours after injection of streptozotocin and the increased number of tubular structures in the portal region of the liver parenchyma occurring at the end of the observation period may have been a result of a cytotoxic effect Streptozotocin is known to possess carcinogenic properties and is reported to cause renal and pancreatic tumours (*Arison* & *Feudal* 1967 *Rakieten et al* 1968, *Steiner et al* 1970) No renal or pancreatic tumours were observed in the Chinese hamsters however, after streptozotocin injection

Marked parenchymatous degeneration with atrophy of tubular cells and secondary cystic dilatation of the lumina of the kidney tubulus has been observed in duct ligated and alloxan treated rats (*Vargas et al* 1970 *Edström* 1973) No appreciable changes in the kidneys of alloxan treated Chinese hamsters have been reported (*Boquist* 1968) In Chinese hamsters with spontaneous hereditary diabetes, hydronephrosis as well as tubular dilatation with tubular epithelial degeneration and intercapillary glomerulosclerosis have been described (*Meier* & *Yerganian* 1959) The renal changes observed after streptozotocin injection in Chinese hamsters most resemble those found in alloxan diabetic and duct ligated rats The cause of these changes is not clear, but the diabetic state may play a role by interfering with normal tubular regeneration (*Vargas et al* 1970) No relevant answers to this question are offered by the present experimental results It may be noted, however that no diabetic glomerulosclerosis appeared in the streptozotocin injected Chinese hamsters

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Supported by grants from the *Swedish Medical Research Council* (Project Nos 107 and 718) and the *Swedish Diabetes Association*

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THE ABILITY OF VARIOUS INSULINS AND INSULIN FRAGMENTS TO INHIBIT THE ANGIOTENSIN I CONVERTING ENZYME

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A variety of insulins and insulin fragments were shown to be active as inhibitors of angiotensin I converting enzyme from guinea pig plasma when tested *in vitro* Angiotensin I was used as substrate and the consumption rate of angiotensin I as well as the generation rate of angiotensin II were measured by means of specific radioimmunoassays for angiotensin I and II The B chain of insulin from different species possessed about the same inhibitory capacity as the whole insulin molecule In an attempt to locate the active part of the sequence of the B chain, the five peptides which resulted from tryptic digestion of the duck insulin B chain were studied They were however, all active as inhibitors and on molar basis equal to or slightly more potent than the B chain and insulin This demonstrates the unspecific nature of converting enzyme Its affinity for insulin and its peptide fragments were, however, two to four orders of magnitude lower than it was for the most potent inhibitor described so far, the synthetic nonapeptide SQ 20 881 This difference was confirmed in *in vivo* experiments.

The inhibitors of angiotensin I converting enzyme can be divided into non peptide and peptide inhibitors, (for literature see *Bakhle* 1974) The most potent peptide inhibitor known so far is a nonapeptide >Glu Trp Pro Arg Pro Glu-Ile-Pro Pro It is found in the venom of Bothrops jararaca and when produced synthetically it is designated SQ 20 881 About the same degree of inhibition was obtained by *Igic et al* (1972) using insulin as well as the B-chain of insulin, but *Sander et al* (1971) found no inhibition with concentrations of insulin 50 fold greater than those used by *Igic et al*

The aim of this study was to examine the ability of various insulins and insulin fragments to inhibit the converting enzyme The main reason why these studies were performed was that if a strong inhibitor of converting enzyme was found among these substances it might be developed into a drug that might be useful in the treatment of hypertension As such an inhibitor should lack the effect of insulin on the carbohydrate metabolism it was found of special interest to study the insulin B chain, but also peptides isolated after tryptic digestion of the duck insulin B chain (*Markussen & Sundby* 1972) and some commercial insulin preparations were included in the study For comparison, the blocking effect of the above mentioned nonapeptide SQ 20 881 was studied

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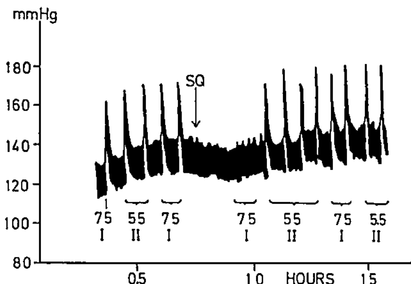


Fig. 1. Results of an *in vivo* experiment in which equipressor responses were obtained with 7.5 ng angiotensin I (marked I) and 5.5 ng angiotensin II (marked II). After i.v. injection of 1.9 mg of the nonapeptide SQ 20.881 per kg b.w. (marked SQ on the figure), the pressor response to 7.5 ng angiotensin was abolished while response to angiotensin II remained unaltered, and it was found that the dose of angiotensin I had to be increased 10 fold in order to re-establish the equipressor responses of angiotensin I and II.

MATERIALS AND METHODS

I. *In vivo* experiments were performed on female Wistar S.P.F. rats, most of which weighed about 100–150 g. They were anaesthetized with 15 mg nactin per 100 g b.w. and had one carotid artery cannulated for the recording of blood pressure, using a Tybjærg Hansen transducer and a Servogor 511 recorder. Determination of the pressor response to angiotensin I and II showed that angiotensin I had about 60 per cent (range in 10 rats 50 to 80 per cent) of the pressor effect of angiotensin II. After repeated injections of equipressor doses of the two angiotensins, the effect of the potential blocker was tested. In experiments in which the ratio between the two angiotensins was changed, the degree of blockade was determined by augmentation of the dose of angiotensin I until equipressor response of the two angiotensins was again obtained (Fig. 1).

II. *In vitro* experiments were performed by determination of the blocking effect of the various insulins and insulin fragments on the conversion of angiotensin I to angiotensin II in incubation mixtures consisting of 1) a converting enzyme preparation, 2) an angiotensin I solution in a buffer system to which 3) the potential inhibitor was added. The incubation was carried out in glass tubes at 37° C for 30 min., shaking being used in experiments with insulin preparations in which the insulin was in suspension, such as Insulin Lente® and Insulin Retard®. The standard incubation mixture consisted of 1) 5 µl of a converting enzyme preparation (a 1 per cent dilution of normal guineapig serum, which was found free of angiotensinase effect in this dilution), 2) 20 µl of a solution of 1.2 ng 1-Asp, 5-Ileu angiotensin I (Bio Schwarz Lot 6910) in a Tris/HCl buffer pH 7.5, containing 1 per cent angiotensi nase free human albumin (from the State Serum Institute, Copenhagen) and 3) 5 µl of either the insulin preparation or other potential inhi or for control b) the Tris/Alb-buffer. The I effect of the preparations was determin comparison of the angiotensin I conte and after a 30 min.s incubation at 37° C

The angiotensin I concentration in th was in all experiments determined immuno assay (*Poulsen & Jorgensen* to verify the specificity of the met decrease in angiotensin I and the angiotensin II during the incub 1971) were measured by respective assays in some experiments. As results were very similar.

The content of Cl ions in the *min buffer* was sufficient for the to be active. Further addition of 200 mM NaCl was found to conversion.

The relatively high concentrat of the insulin preparations

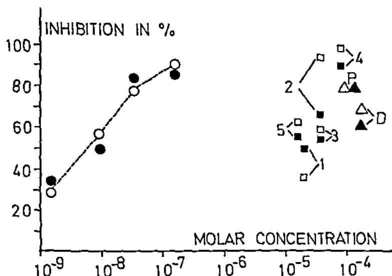


Fig 2 A comparison of the *degree of inhibition of the converting enzyme measured either by the de crease in angiotensin I* (open symbols) *or by the formation of angiotensin II* (closed symbols) The figure includes studies performed with the *nonapeptide* (○ and ●), the amino-ethylated pork (marked P) and duck (marked D) *B chains* of insulin (△ and ▲) and *tryptic peptides* of the duck insulin B-chain (□ and ■), the letters and numbers of which relate to those mentioned in the section materials and methods and in the text to fig 3 The ordinate gives the percentual degree of inhibition the abscissa the molar concentrations of the inhibitors

non specific displacement of the standard curve in the radio-immuno assay for angiotensin In such cases, correction for this displacement was obtained by including insulin in the standards

Substances tested for blocking effect The nonapeptide, insulin beef pro-insulin insulin B-chain A+B chain S sulphonate preparation and 5 tryptic peptides from the B chain The *nonapeptide* was the SQUIBB preparation 20 881 The *insulins* were two batches of crystalline pork insulin (lot 823044 and 1968) (marked P1 and P2 in Fig 3) Insulin Lente® (marked L) and Insulin Actrapid® (marked A) from NOVO a pork insulin from Schwarz Mann (lot NOZZ 1123) (marked S in Fig 3) and Insulin Neutral® and Insulin Retard® (marked N and R) from Leo The beef *pro insulin* was obtained from NOVO (lot 17370) The insulin *B chains* were an oxidized (phenyl analine) preparation (Lot Y 3980) from Schwarz Mann (marked S) an oxidized form from beef insulin (Lot 72331 11) from Mannheim Boehringer (marked B) and amino-ethylated pork and duck B chains (marked P and D in Fig 3) from NOVO The duck insulin B chain has been shown by *Markussen & Sundby* (1973) to have an amino acid sequence which is very similar to that of the B chain from human insulin that of the duck chain being Ala Ala Asn Gln His Leu Cys/ Gly Ser His Leu Val Glu Ala Leu Tyr/Leu Val Cys/ Gly Glu Arg/Gly Phe Phe-Tyr Ser Pro Lys/Thr This B-chain was by *Markussen & Sundby* (1973) split by tryptic digestion into five peptides (and an amino acid) The inhibitory effects of the peptides on angiotensin I converting enzyme are included in the present study, the five peptides (marked 1 to 5 in Fig 2 and 3) containing amino acid sequences 1 7, 8–16, 17–19 20 22 and 23 to 29 respectively This splitting of the B chain is marked by slanting lines in the amino acid sequence given above The S sulphonates of the insulin A and B-chains were prepared as described by *Du et al* (1965) The A and B chain

TABLE 1 *Inhibition of Angiotensin I-Induced Pressor Responses*

Preparation	Dose (mg/kg)	Mean max inhibition (in per cent)	Number of tests
SQ 20 881	1–3	80 95	4
	0 3–0 4	50–85	4
Insulin B-chain			
1) NOVO	5–32	0	8
2) Schwarz Mann	30–200	0	9
3) Boehringer	53–110	0	3
Insulin A + B chain S-sulphonate NOVO	220–610	0	8

S-sulphonates were present in an equimolar mixture The B chain S-sulphonate was isolated from the mixture by gel filtration on a column of Sephadex G 75 in 50 per cent acetic acid as described by *Varandani* (1966)

RESULTS

I In vivo Experiments

The *in vivo* experiments included studies of the ability of the nonapeptide SQ 20 881, various B-chain preparations and an A + B-chain sulphonate (NOVO) to block the conversion of intravenously *injected* angiotensin I to angiotensin II in anaesthetized rats The results are given in Table 1 which shows that, while 0 3 mg/kg b w of the nonapeptide gave a marked *inhibition*, no inhibition was found if 100–700-fold this dose of the various insulin B-chains or 1000–2000-fold this dose of the A + B-chain sulphonate were used

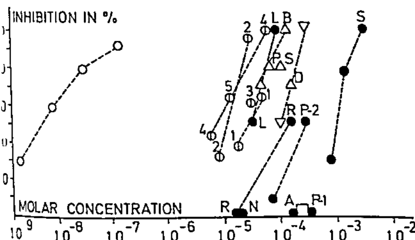
II In vitro Experiments

The results of the *in vitro* experiments are given in Fig 3 *The nonapeptide SQ 20 881* was found to be a strong inhibitor of the converting enzyme a concentration of 1 5 × 10⁻⁷ M giving 85–100 per cent inhibition Fig 2 shows the very similar dose-response curves obtained by measurements of 1) the decrease in angiotensin I concentration and 2) the increase in angiotensin II formation during the 30-minute incubations

Fig 3 further shows that the *B chain of ox, pork and duck insulin* can inhibit the converting enzyme but that this is only the case when their concentrations are at least 1000-fold higher than those used in the experiments with the nonapeptide

The molar concentration of *insulin* necessary to obtain inhibition was about the same for one of the preparations (the Insulin Lente®) and for the other four it was somewhat higher than that of the insulin B-chains Two preparations Insulin Neutral® (marked N in Fig 3) and Insulin Actrapid® (marked A) were without effect in the concentrations used It was further found that ox *pro-insulin* in the concentration given in Fig 3 was incapaple of inhibiting the converting enzyme

The results of the study of the inhibiting ability of *the peptides of the duck insulin B-chain* isolated after tryptic digestion are also given in Fig 3 It appears that they are all able to inhibit the converting enzyme and that although there is some difference between the concentrations needed to reach the same degree of inhibition they are all in the 10^{-4}–10^{-5} molar range The specificity of this inhibition was ensured by control experiments which are shown in Fig 2, in which the degree of inhibition of the converting enzyme was measured both by the decrease in angiotensin I and by the formation of angiotensin II The results obtained by the two methods are seen to be very similar



g 3 *Relation between the percentual degree of inhibition of the converting enzyme* (ordinate) *and e molar concentration of the different preparations* the nonapeptide SQ 20 881 (O) the insulin ●) and B-chain (Δ) preparations, and A and B-chain sulphonate (∇), the proinsulin preparation □) and finally the tryptic peptides obtained from the duck insulin B chain (Φ) The letters and umbers given above symbols indicate the different preparations in these groups for the *insulins* L nsulin Lente®, A – Insulin Actrapid®, P1 and P2 – two NOVO pork insulins, N – Insulin Neutral®, Ins In Retard®, S a pork insulin from Schwarz Mann For the *B chains* B = a Boehringer and a Schwarz Mann preparation, while P and O are amino-ethylated pork and duck insulin B-chains om NOVO As regards the numbers above the symbols for the *tryptic peptides from duck insulin chain* 1 indicates the heptapeptide Ala Ala Asn Gln His Leu Cys 2 the nonapeptide Gly Ser His eu Val Glu Ala Leu Tyr, 3 and 4 the tripeptides Leu Val Cys and Gly Glu Arg, respectively, and 5 e heptapeptide Gly Phe Phe Tyr Ser Pro Lys Where the effect of more than one concentration of a reparation was studied the symbols are connected with a dashed line Each value is based on at least ree experiments

DISCUSSION

onverting enzyme is able to cleave a wide ariety of peptides and the peptide inhibitors re as diverse as the substrates (see *Bakhle* 974) The present study has confirmed this s will be seen from the results of the *in itro* studies in Fig 3 The figure further hows that although various insulins and inulin fragments are able to inhibit the con erting enzyme they are much less effective nhibitors than the nonapeptide SQ 20 881 This finding is also confirmed by the results of the *in vivo* studies given in Table 1 This marked difference in degree of inhibition contrasts with the results obtained by *Igi et al* (1972) who, using *in vivo* as well as *in vitro* assays, found about the same inhibitory capacity of the nonapeptide, insulin and the insulin B chain The reason for this discrepancy between their results and those obtained in the present study is unknown

Fig 3 further shows that, although some *insulin* preparations and *proinsulin* do inhibit the converting enzyme (in the concentrations used) most of them are active inhibitors when used in molar c from 10^{-3} to 10^{-4} The Insulin

found to be the most active, which could be due to its higher content of zinc, which has been shown to inhibit converting enzyme (*Bakhle* & *Reynard* 1971) The differences in activity of the other insulin preparations may be due to differences in accessible sites available on the surface of the molecules The *insulin B-chains* and especially the *tryptic peptides obtained from the duck B-chain* were active in concentrations lower than those needed for inhibition using the whole insulin molecule The above mentioned diversity of the peptide inhibitors is confirmed by the finding that all five peptides isolated from the B chain were active, the differences in degree of their activity being small as compared with the field covered by all the inhibitors studied in the present investigation (Fig 3)

This study was supported by grants from the *Foundation of the Insurance Companies of 1952*, the *Danish Heart Foundation* and *King Christian X s Foundation* The authors are also grateful to Dr *F F Giarusso, The Squibb Institute* for Medical Research, New Jersey, USA for a generous gift of the nonapeptide and to the head of the *NOVO Insulin Laboratories*, *J Brange* and *H* , the *NOVO Research Institute*, Copenhagen

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AN ATTEMPT TO USE THE WHO TYPING IN THE HISTOLOGICAL CLASSIFICATION OF LUNG CARCINOMAS

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An earlier lung carcinoma material collected during 1968–1971 was re examined The series comprised 175 cases, 91 (52 per cent) of which were detected by biopsy and 84 (48 per cent) at autopsy The significance of the results was evaluated in the light of the clinical course of the disease, mainly in the light of the survival Epidermoid carcinomas were classified into two groups, one comprising carcinomas with high or moderate differentiation and another comprising those with slight differentiation, which were then dealt with as separate categories The distribution was as follows 68 (38 85 per cent) highly or moderately differentiated epidermoid carcinomas, including 6 of the clear cell type which are here classified as belonging to this group, and 27 (15 44 per cent) slightly differentiated epidermoid carcinomas, making up a total of 95 (54 29 per cent) Parvo cellular anaplastic carcinomas numbered 52 (29 72 per cent) and adenocarcinomas 15 (8 57 per cent) among which 12 (6 86 per cent) were bronchogenic and 3 (1 71 per cent) bronchiolo-alveolar Large cell carcinoma, mucous gland carcinoma, combined epidermoid adenocarcinoma and carcinosarcoma each occurred once (0 57 per cent) There were a further 7 (4 0 per cent) unclassifiable cases and 2 (1 14 per cent) mesotheliomas Survival in the two groups of epidermoid carcinomas was seen to differ significantly As regards the 3 patients with clear cell carcinomas survival was notably good It was difficult histologically to draw a line between the slightly differentiated epidermoid carcinoma and the fusiform and polygonal subtypes of the anaplastic parvocellular carcinoma Nor was there any significant difference in survival in these groups

he impetus to this work was given by the bservation that the borderline cases of epi ermoid carcinoma seem vaguely delimited

In each of the large series of lung carcinomas collected by *Kreyberg & Saxen* (1, 2), he proportion of epidermoid carcinomas was lightly over 50 per cent in each The WHO istological classification (3) is relatively easy o apply to the highly differentiated types the lifficulties encountered in slightly differentiated cases have been discussed by *Kreyberg* later on (4) In his discussion *Kreyberg* also dealt with the biological relation between the clear-cell subtype of the large cell carcinoma and the epidermoid carcinoma

The histological classification of lung carcinomas is rendered even more difficult by the fact that the contents of the classifications employed by different authors vary considerably as regards the slightly differentiated carcinomas In the investigation by *Feinstein et al*, observer variability in the cases of slightly differentiated epidermoid carcinomas and adenocarcinomas was 40–42 per cent (5), and it was similarly great in the work

Received 25 iii 74 Accepted 24 vi 74

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of *Kutschera* & *Salzer* (6). The proportion of large cell carcinomas varies greatly in the different series. In the series collected by *Hallgrimsson* (7), for example, it was as high as 14.8 per cent.

The validity of the histological classification of lung carcinomas recommended by the WHO was evaluated on the basis of the clinical course of the disease, mainly on the basis of the survival.

MATERIAL AND METHODS

The series comprised the 181 cases of lung carcinoma examined in the Department of Pathology, University of Oulu, during 1968–1971. Among the 181 samples were 6 which were either too small or necrotic, and the final series consequently comprised 175 samples.

The autopsy cases were chosen in such a way that the main cause of death was lung carcinoma. The follow up period was 2–6 years, and survival was controlled at 3 month intervals. Follow up was started at the time of the final diagnosis. In cases where the diagnosis was not made until at autopsy, follow up was started at the time when the patient was admitted to hospital. The age and sex of the patient, the duration of symptoms, and the method of treatment were also taken into account in the analysis.

Among the 175 cases, 91 (52 per cent) were detected by biopsy and 84 (48 per cent) at autopsy. The biopsies included 30 surgical preparations, 50 bronchoscopies, 8 mediastinoscopies, and 3 needle biopsies. The autopsy series included 36 cases in which the diagnosis had not been made until at autopsy. The whole series contained only 4 cases from which a biopsy sample was obtained as well as one sample obtained at autopsy.

The original sections stained with haematoxylin eosin were re-examined, and new sections were made and stained for mucin and keratin according to the combined method of *Kreyberg* (Histological typing of lung tumours, WHO 1967, pp. 27–28). Ladewig's anilin blueorange fuchsin method was employed to visualize intercellular bridges and keratin. Where a carcinoid tumour was suspected, the sections were stained according to Fontana Masson's silver method to visualize the argentaffin granules. In connection with this re-cut haematoxylin eosin sections were made in order to check the information obtained from the original HE sections.

The differences between the groups as regards statistical significance were calculated using the X^2 test, taking into account the effect of two variables by crosstabulation (Documentary Geigy 7th edition, Scientific tables, pp. 36–39). The inter group comparisons of survival were made halfway through each 3 month period.

TABLE 1. *Histological Types of Lung Carcinoma during the Period 1968–71. Mean ages of Patients in the Three Largest Groups. Department of Pathology, University of Oulu, Finland*

Histological type	Number		Total		Mean ages of patients
	males	females	number	per cent	
Well or moderately differentiated epidermoid carcinoma including clear cell carcinoma	64	4	68	38.85	59.38 ± 9.77
Slightly diff. epid. ca.	26	1	27	15.44	60.59 ± 8.65
Epidermoid carcinoma. Total	90	5	95	54.29	
Small cell anaplastic carcinoma	46	6	52	29.72	59.5 ± 9.7
Large cell carcinoma	1		1	0.57	
Adenocarcinoma	14	1	15	8.57	58.80 ± 13.02
Combined epidermoid adenocarcinoma	1	–	1	0.57	
Bronchial gland carcinoma	1	–	1	0.57	
Carcinosarcoma	1	–	1	0.57	
Unclassified carcinoma	6	1	7	4.00	
Mesothelioma	1	1	2	1.14	
Total	161	14	175	100%	

TABLE 2 *The Distribution of the Histological Types and WHO Subtypes in the Three Largest Groups of Biopsy and Autopsy samples*

Sample	Parvocellular carcinoma Number of cases per subtype					Epidermoid carcinoma Number of cases per differentiation group			Adenocarcinoma Number of cases per subtype		
	Fusiform	Polygonal	Oat cell	Others	Total	Well or moderately	Slightly	Total	Bronchogenic	Bronchiolo-alveolar	Total
Biopsy	4	12	1	2	19	52	12	64	3	1	4
Autopsy	3	7	21	2	33	16	15	31	9	2	11
Total	7	19	22	4	52	68	27	95	12	3	15

RESULTS

Grouping According to the Histological Findings

Epidermoid carcinomas were divided into two groups on the basis of the degree of differentation: highly or moderately differentiated carcinomas and slightly differentiated carcinomas. The latter group included the cases where presence of intercellular bridges and keratin remained uncertain but which displayed solid cellular composites typical of epidermoid carcinoma or epidermoid features called corps ronds. The slightly differentiated type sometimes contained material staining weakly like mucus, which was considered a secondary degenerative or metaplastic phenomenon.

The clear cell carcinomas in which no intercellular bridges could be distinguished constituted a special group. At least some of them, however, showed signs of epidermoid cell growth. These cases were at first considered a separate group.

Frequencies of the Histological Types and Comparison of the Biopsy and Autopsy Series

The typological distribution is shown in Table 1. The largest group consisted of 95 (54.29 per cent) epidermoid carcinomas, which 68 (38.85 per cent) were highly or moderately differentiated, including 6 (3.43 per cent) clear cell carcinomas.

Table 2 shows the distribution of the histological types and WHO subtypes in the three largest groups of biopsy and autopsy samples. The only large cell carcinoma in the series was an autopsy sample and it was of the giant cell subtype. The only mucous gland carcinoma, which was of the mucoepidermoid subtype, was similarly an autopsy sample.

Among the 36 cases which were not diagnosed until at autopsy were 18 parvocellular anaplastic carcinomas, of which 12 were of the oat-cell subtype. Eight out of a total of 36 were well or moderately differentiated epidermoid carcinomas, while 4 were poorly differentiated epidermoid carcinomas.

The data available were not sufficiently detailed to permit a comparison of the size of tumours, their expansion and the possible ulceration of the bronchial mucous membrane. In the autopsy series the epidermoid carcinoma displayed extensive metastases less frequently than the anaplastic parvocellular carcinoma. The oat cell carcinoma had metastasized extensively in each case.

Age Distribution and the Duration of Symptoms before the Initiation of Treatment

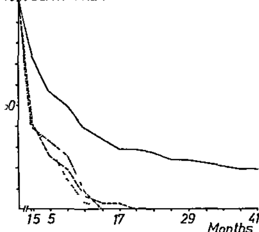
The mean ages of the patients in the three largest groups are given in Table 1. As can be seen, there is no significant difference in the mean age of patients with the three main types of carcinoma. The average period over which symptoms had been experienced was 3.6 months, being somewhat longer in the group of epidermoid carcinomas than in the other groups.

Survival Rate

The clear cell group was small and the survival rate of the patients in this group was comparable with that of the subjects with highly or moderately differentiated epidermoid carcinoma. Since there were also histological signs of epidermoid cell growth in some clear cell carcinomas, these cases were included in the group of highly or moderately differentiated epidermoid carcinomas.

In the total series, the number of patients still alive was 12. They all belonged to the highly or moderately differentiated epidermoid group; three of them had a clear-cell carcinoma. The surviving patients have been followed for 2–6 years.

The survival rate of the patients with highly or moderately differentiated epidermoid carcinoma, slightly differentiated epidermoid carcinoma, parvocellular anaplastic carcinoma and adenocarcinoma reckoned from the time of the final diagnosis onwards is given in Fig. 1. It appears that the survival is clearly best in cases of highly or moderately differentiated epidermoid carcinomas



g 1 Survival of patients with highly or mode tely differentiated epidermoid carcinoma includ g clear cell carcinoma, slightly differentiated epi rmoid carcinoma, small-cell anaplastic carci oma, and adenocarcinoma.

——— Highly or moderately differentiated epi dermoid carcinoma (68 cases)
Small cell anaplastic carcinoma (52 cases)
— —— Adenocarcinoma (15 cases)
Slightly differentiated epidermoid carci noma (27 cases)

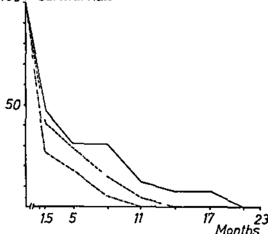


Fig 2 Survival of patients with the fusiform or polygonal subtype of small-cell anaplastic carci noma, oat cell subtype, and slightly differentiated epidermoid carcinoma

——— Polygonal and fusiform cell type (26 cases)
– – Slightly differentiated epidermoid carci noma (27 cases)
—·—·— Oat cell carcinoma (22 cases)

he difference from the survival in cases of lightly differentiated epidermoid carcinoma nd parvocellular carcinoma is highly signi icant ($p<0.001$) Compared with the adeno arcinoma the survival of the well or mode ately differentiated epidermoid group was ignificantly better ($p<0.05$) The survival ates in cases of the slightly differentiated pidermoid carcinoma the adenocarcinoma nd the parvocellular carcinoma did not diff r significantly ($p<0.05$)

It can be seen from Fig 2 that the survival in cases of the slightly differentiated epider moid carcinoma was not significantly diffe-rent from the survival in cases of the poly-gonal and fusiform subtypes of the parvo cellular anaplastic carcinoma ($p<0.05$) The survival in cases of the polygonal and fusi form subtypes differed significantly from that in cases of the oat cell subtype ($p<0.001$) The survival rates in cases of the slightly dif ferentiated epidermoid carcinoma and the oat cell carcinoma did not differ significant-ly from each other in the present series ($p<0.05$)

Comparison of Therapeutic Methods

Table 3 shows the methods used in the treatment of the main histological types of lung carcinoma The different therapeutic methods used in cases of epidermoid carci-noma are compared in Fig 3 It shows that in cases merely receiving operative treatment the survival is significantly better than that in the group of patients receiving both opera tive treatment and radiotherapy ($p<0.05$) The survival of those who received only radiotherapy was significantly better than that of the patients who did not receive any therapy ($p<0.001$) Of the 12 surviving cases, 8 were epidermoid carcinomas treated surgically exclusively and 4 epidermoid carci nomas which had received both operative treatment and radiotherapy We do not at-tempt to draw any further conclusions, be-cause the choice of the therapeutic method

TABLE 3 *Therapy Groups according to Histological Tumour Type Epidermoid Carcinoma Small Cell Carcinoma, and Adenocarcinoma*

	Epidermoid carcinoma	Small cell carcinoma	Adeno-carcinoma
Number of patients	95	52	15
Therapy group	%	%	%
Resected	16 8	5 8	20 0
Resected and radiated	15 9	–	20 0
Radiated	36 8	48 1	6 7
Non treated	30 5	46 1	53 3

and the outcome of treatment depend on several factors, such as the extent of the carcinoma and the possible concurrent diseases of the patient

Comparison of Staining Methods

Haematoxylin-eosin proved to be the best general staining method, *Kreyberg*'s method was necessary for visualizing the mucus, and Ladewig staining revealed best the intercellular bridges and keratin The re-cut HE-section gave a result different from that obtained from the original HE-section in 4 cases

DISCUSSION

Comparison of the Frequencies of the Different Histological Types

The results obtained in cases of epidermoid carcinoma were parallel to those reported by *Saxen* & *Kreyberg* (1, 2) Epidermoid carcinomas were slightly more numerous in the present material than in the other two (*Kreyberg* 51 83 per cent *Saxen* 53 69 per cent, present series 54 29 per cent) Anaplastic parvocellular carcinomas were clearly least frequent in Kreyberg's series (*Kreyberg* 17 67 per cent, *Saxen* 25 16 per cent present series 29 72 per cent), while adenocarcinomas were considerably more numerous (*Kreyberg* 19 67 per cent *Saxen* 8 17 per cent, present series 8 57 per cent) Carcinoid tumours were numerous in *Kreyberg*'s material (9 17 per cent), but we had no occurrences of this type We consider the histological diagnosis of carcinoid tumours very difficult, unless argentaffin granules are present

The Significance of Anaplasia in Epidermoid Carcinoma and the Limits of the Epidermoid Group

According to the WHO histological classification, epidermoid carcinomas are characterized by intercellular bridges or keratin We know from experience that the identification of intercellular bridges in the slightly differentiated type requires a long and careful examination It was therefore justifiable to make the classificatory criteria for the epidermoid carcinoma somewhat wider

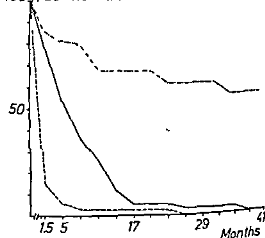


Fig 3 Survival of patients with resected, resected and radiated, merely radiated and non treated epidermoid carcinoma (all grades of differentiation included)

Resected patients (16 cases)
Resected and radiated patients (15 cases)
Radiated patients (35 cases)
Non treated patients (29 cases)

Owing to the smallness of the series, no at importance can be attached to the sur- al rates It is useful, however, to note the ference in survival according to degrees of ferentiation of the epidermoid carcinoma, the one hand, and the similarity in survi- l in the cases of the slightly differentiated idermoid carcinoma and the fusiform and lygonal subtypes of the anaplastic parvo lular carcinoma, on the other hand

The good survival in cases of the clear-cell rcinomas, here included in the group of hly or moderately differentiated epider oid carcinomas, is also worthy of mention, spite the small number of cases *Spencer* scribes this type as being slow in growth) *Eck et al* divide the epidermoid carci omas into two types the clear-cell non ratinizing type and the keratinizing type) *Kreyberg* (4) discusses the biological lation between the clear-cell carcinoma and e epidermoid carcinoma "Subtype IV, 4 arge cell carcinoma clear cell subtype) pecially shows many features resembling e tumours of 'cellules a manteau' of French hool some of the tumours possibly biologi- ally related to epidermoid carcinomas"

osition of Oat-cell Carcinoma in he Classification

It turned out that the oat cell carcinomas ere mainly diagnosed at autopsy, as *Spencer* 8) and *Kreyberg* (10) have also pointed out ccording to the present work, the reason or this would seem to lie, at least partly, in he fact that the oat-cell carcinoma progresses vere rapidly, which is why the final diagnosis s often not made until at autopsy

During the re-examination of the 4 cases rom which both biopsy and autopsy samples vere obtained, the two samples were con trasted in each case In two of them the biopsy subtypes polygonal and fusiform turned out to be oat-cell in the autopsy sample

It may be that because of the deteriora- tion of cellular coherence and dissolution of cytoplasm due to autolysis oat cell carcinoma is more frequently diagnosed at autopsy than at biopsy

Since, moreover, this work is not an epide- miological one, the material is somewhat se- lective

CONCLUSION

The fact that the material is small detracts from the value of the findings They seem to suggest, however, that it would be expedient to treat the slightly differentiated epidermoid carcinomas and the highly or moderately dif- ferentiated epidermoid carcinomas as sepa rate groups, as *Feinstein et al* (5) have re commended It might be reasonable to in- clude the slightly differentiated epidermoid carcinomas and the fusiform and polygonal parvocellular carcinomas in one group, be cause their differential diagnosis is histologi- cally difficult, and there is no significant dif ferences between them as regards survival

Difficulties and ambiguities are hence still encountered in the application of the WHO classification

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BRIEF REPORT

SIZE AND DENSITY OF THE MITOCHONDRIA IN PURKINJE CELLS OF RATS AFTER THIOPHEN INTOXICATION, AS MEASURED BY IMAGE ANALYSING SYSTEM

R Albrechtsen, N H Diemer and M H Nielsen

iophen (C_4H_6S) is one of a large number of xious agents which attack restricted areas of the rvous system in a selective manner, but its mode action is unknown (2, 5, 11) In the cerebellum rats a destruction of small neurons in the gran ır layer occurs after 5 10 days of daily sub taneous injections of 0 4 ml thiophen The Pur nje cells may also be affected, as they display toplasmic vacuoles 2 weeks after the initiation of e injections, and after 8 weeks there is dilatation the endoplasmatic reticulum with rounding of e mitochondria (5, 10)

Enzyme histochemical investigations have re aled a reduction of NADH tetrazolium reductase NADH Tr) activity in the Purkinje cells of all imals with thiophen intoxication (1) The de ease in NADH-Tr activity was most pronounced ter the period of maximal necrosis in the gran ar layer, i e thirty days after the first injection thiophen The enzymatic change was therefore sumed to be secondary to the granule cell in lvement, possibly by a transneuronal mechanism 1, 4) Since NADH Tr is 'structurally bound' to itochondria (8) we found it relevant to inves gate the mitochondrial system during changes of ADH Tr activity

The purpose of the present study was to de rmine whether there were any changes in the ze and density of the mitochondria in Purkinje lls during thiophen intoxication The area of the itochondria was determined by means of an elec onic image analysing system

Received 4 x 74 Accepted 4 x 74

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Material and Methods

Albino rats aged 4 months, weighing about 200 g were given daily subcutaneous injections of 0 4 ml thiophen (Merck, Darmstadt No 8157) The site of injections was varied to minimize local tissue damage Two untreated albino rats of the same age and weight served as controls To avoid differences caused by diurnal changes in cell function all ani mals were killed at the same time of the day

Thirty days after the initial injection two treated rats and two controls were anaesthetized with sodium pentobarbital (11 mg/100 g body weight) and artificially respirated Vascular perfusion was performed using 6 per cent glutaraldehyde in iso tonic phosphate buffer (pH 7 5) with 3 per cent Dextran T 70, and tissue blocks were post fixed in osmium tetraoxide, dehydrated in alcohol and em bedded in Vestopal W (7) Purkinje cells from both cerebellar hemispheres were identified by light microscopy of 1 micron sections, and ultrathin (500 800 Å) sections were stained with magnesium uranyl acetate and lead citrate and examined in a Siemens Elmiscope I Electron micrographs (total magnification 33,000) of the cytoplasm of 12 16 Purkinje cells in each group were obtained Nuclear and plasma membranes were marked with red ink and the total area of all cross sectioned mitochondria was coloured black The area of the mitochondria was measured on a Leitz Classimat (6) This system transmits the electron micrograph via a television camera to a monitor screen, the image is analysed by an electronic computer and the data stored on punch tape Area determinations with Leitz Classimat have shown that deviations in reproducibility due to the tuning in of the instru ment are below ± 3 3 per cent (3) By using a grey tone discriminator only the black stained mito chondria were selected for measurement An elec tronic mask made it possible to determine the area of one single mitochondria at a time (Fig 1) The total area of the Purkinje cell cytoplasm was de termined by means of compensating planimetry

TABLE 1 *Mean Areas Density and Ratio Mitochondrial Area/Cytoplasmic Area in the Thiophen treated Rats and Controls*

	Mean cross sectional mitochondrial area (micron²)	Number of mitochondria per 100 micron² cytoplasmic area	Mitochondrial area as percentage of cytoplasmic area	Number of mitochondria in each group
Thiophen treated rats	0.052 ± 0.013	105.8 ± 21.8	5.3 ± 1.1	1452
Control rats	0.037 ± 0.005	140.4 ± 18.2	5.2 ± 0.7	943
	$p < 0.0005$	$p < 0.001$	$p > 0.25$	

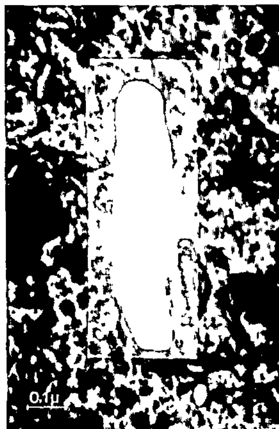


Fig 1 Classimat monitor screen on electron micrograph with marked mitochondrion within displayed mask

(Koizumi type kp 27) Student's t test was used for statistical analysis

Results

The areas of 2395 mitochondria from 53 electron micrographs of Purkinje cell cytoplasm were measured (Table I) The mitochondria of thiophen intoxicated rats were significantly larger than the mitochondria of control animals with a mean cross sectional area of individual mitochondria of 0.052 micron² and 0.037 micron², respectively ($p < 0.0005$) The density of mitochondria in the Purkinje cells of intoxicated rats was reduced from the normal value of 140.4 (number of mitochondria per 100 micron² cytoplasmic area) to 105.8 ($p < 0.001$), but in both groups the ratio mitochondrial area to cytoplasmic area was identical The mitochondria were found to occupy 5 per cent of the cytoplasmic area of the cross-sectioned Purkinje cells

Comments

The results demonstrate that changes due to thiophen intoxication also affect neurons outside the granular layer in the mitochondrial system in the Purkinje cells The morphological changes are inconspicuous, but an increase in mitochondrial size has also been described in other cells often as a consequence of damage to mitochondrial enzyme systems (9) By using an image analysing computer, however, we have shown that the increase in mitochondrial size due to thiophen intoxication was compensated by a decrease in mitochondrial density, whereby the cells maintained the same mitochondrial/cytoplasmic area ratio

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